DOCTORAL THESIS

Identification of new biomarkers to predict the risk of Type 2 Diabetes Mellitus in patients with Cardiovascular Disease

CORDIOPREV -DIAB study

ROSA JIMÉNEZ LUCENA

Thesis by Compendium of Articles with International Mention

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TITULO: *Identification of new biomarkers to predict the risk of Type 2 Diabetes Mellitus in patients with Cardiovascular Disease: CORDIOPREV-DIAB study*

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UNIVERSIDAD DE CÓRDOBA
DEPARTAMENTO DE MEDICINA

IDENTIFICATION OF NEW BIOMARKERS TO PREDICT THE RISK OF TYPE 2 DIABETES MELLITUS IN PATIENTS WITH CARDIOVASCULAR DISEASE: CORDIOPREV-DIAB STUDY

Trabajo presentado por

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Licenciada en Farmacia, para optar al grado de
Doctor por la Universidad de Córdoba

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Programa de Doctorado: Biomedicina

Dirigido por

Prof. Dr. José López Miranda

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TITULO DE LA TESIS: Identificación de nuevos biomarcadores para predecir el riesgo de diabetes mellitus tipo 2 en pacientes con enfermedad cardiovascular. Estudio CORDIOPREV-DIAB.

DOCTORANDA: Rosa Jiménez Lucena

INFORME RAZONADO DEL/LOS DIRECTOR/ES DE LA TESIS.

El trabajo de tesis realizado por Rosa Jiménez Lucena, bajo nuestra dirección en la Unidad de Lípidos y Arteriosclerosis del Hospital Universitario Reina Sofía/Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC), se basó en la identificación de nuevos biomarcadores para predecir el desarrollo de diabetes mellitus tipo 2 en pacientes con enfermedad coronaria. Los resultados obtenidos responden a los objetivos planteados. En cuanto a su difusión, la doctoranda ha participado en 14 congresos científicos de ámbito Nacional e Internacional, presentando comunicaciones orales y de tipo póster. Así mismo, dichos resultados se han publicado en colaboración con los tutores de tesis y otros colaboradores de nuestro grupo de investigación, lo que le ha permitido a la doctoranda presentar su tesis por compendio de artículos. Las publicaciones derivadas de esta tesis doctoral son las siguientes:

Camargo, A.*, **R. Jimenez-Lucena***, J. F. Alcala-Diaz, O. A. Rangel-Zuniga, S. Garcia-Carpintero, J. Lopez-Moreno, R. Blanco-Rojo, J. Delgado-Lista, P. Perez-Martinez, B. van Ommen, M. M. Malagon, J. M. Ordovas, F. Perez-Jimenez and J. Lopez-Miranda (2018). "*Postprandial endotoxemia may influence the development of type 2 diabetes mellitus: From the CORDIOPREV study.*" Clin Nutr. doi: 10.1016/j.clnu.2018.03.016. **Impact factor: 5.496 (D1).** *equal contributions.

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Además, la generación de una patente titulada "Modelo predictivo para predecir el desarrollo de diabetes mellitus tipo 2 usando miRNAs" con número de registro P201830540.

A nuestro juicio, el trabajo realizado por la doctoranda Rosa Jiménez Lucena reúne los méritos suficientes para ser defendido ante el tribunal correspondiente y poder optar al grado de Doctor por la Universidad de Córdoba. Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 26 de octubre de 2018

Firma de los directores:

Prof. Dr. José López Miranda

Dr. Oriol Alberto Rangel Zúñiga

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"Cada día sabemos más y entendemos menos"
Albert Einstein

"Investigar es ver lo que todo el mundo ha visto, y pensar lo que nadie más ha pensado"
Albert Szent

"El poder de cuestionar es la base de todo progreso"
Indira Gandhi

"A veces sentimos que lo que hacemos es tan solo una gota en el mar, pero el mar sería menos si le faltara una gota"
Madre Teresa de Calcuta

INDEX

ABREVIATURAS

Las abreviaturas más comúnmente utilizadas en el texto se detallan a continuación:

CORDIOPREV: COROnary Diet Intervention with Olive oil and cardiovascular PREVention study

ADA: American Diabetes Association

HbA1c: Hemoglobina glicosilada

GAD: Enzima glutamato decarboxilasa

DMT1: Diabetes mellitus tipo 1

HSP70: Proteínas de choque térmico

TNF: Factor de necrosis tumoral

DMG: Diabetes mellitus gestacional

DMT2: Diabetes mellitus tipo 2

IFG: Alteración de la glucosa plasmática en ayunas (impaired fasting glucose)

IGT: Alteración de la glucosa plasmática a las 2 h del OGTT (impaired glucose tolerance)

FPG: Glucosa plasmática en ayunas (fasting plasma glucosa)

2h PG: Glucosa plasmática tras 2 horas del OGTT

OGTT: Test de sobrecarga oral de glucosa

TFG: Tasa de filtración glomerular

GAD: Glutamato decarboxilasa

HLA: Human leucocyte antigen

NHANES: Encuesta Nacional de Salud y Nutrición

ECV: Enfermedad cardiovascular

OMS: Organización Mundial de la Salud

DPP: Programa de Prevención de la Diabetes

NCDs: Enfermedades no transmisibles (non-communicable diseases)

IMC: Índice de masa corporal

GIP: Polipéptido inhibidor gástrico

GLP1: Péptido 1 similar al glucagón

GSIS: Secreción de insulina estimulada por glucosa

SREBP: Proteína de unión al elemento regulador de esteroles

HNF: Factor nuclear hepático

ER: Estrés del retículo endoplasmático

UPR: Unfolded protein response

ROS: Especies reactivas de oxigeno

UCP2: Proteína de desacoplamiento 2

AGL: Ácidos grasos plasmáticos libres

NEFA: Ácidos grasos no esterificados (non-esterified fatty acids)

DAG: Diacilglicerol

PKC: Proteína quinasa C

NAFLD: Enfermedad hepática no alcohólica

LpL: Lipoprotein lipasa

GLUT2: Transportador de glucosa 2

G6P: Glucosa-6-fosfato

TCA: Ciclo de los acidos tricarboxílicos (Ciclo de Krebs)

DHAP: Dihidroxiacetona

Gly3P: Glycerol-3 fosfato

FFAR: Receptor de ácidos grasos libres

NO: Óxido nítrico

TG: Triglicéridos

QUICKI: Índice cuantitativo de control de sensibilidad a la insulina

HOMA: Homeostasis model assessment

IST: Test de supresión de insulina

FSIVGTT: Minimal model analysis of frequently sampled intravenous glucose tolerance test

OGSI: Índice de sensibilidad oral a la glucosa

ISI: Índice de sensibilidad a la insulina

IGI: Índice insulinogénico

DI: Disposition index

HIRI: Índice de resistencia hepática a la insulina

MISI: Índice de sensibilidad muscular a la insulina

FINDRISC: Finnish Diabetes Risk Score

IEC: Células epiteliales intestinales

TJs: Tight junction proteins

EII: Síndrome del intestino irritable

LPS: Lipopolisacárido

ME: Endotoxemia metabólica

LBP: LPS binding protein

MD-2: Proteína de diferenciación mieloide-2

TLR: Toll like receptor

PRR: Receptores de reconocimiento de patrones

MAMP: Patrones moleculares asociados a microorganismos

CD: Células dendríticas

NF-κB: Factor nuclear kappa B

UTR: Región no traducida

DGCR8: Proteína de la región crítica del gen 8 del síndrome DiGeorge

GTP: RAN guanosina trifosfato

TRBP: Proteína trans-activadora de unión a RNA

RISC: RNA Induced Silencing Complex

Ago2: Argonauta 2

miRNAs: microRNAs

ARNm: ARN mensajero

MVB: Cuerpos multivisiculares

nSMAse2: Esfingomielinasa 2 neutra

Mtpn: Miotrofina

PBMC: Células mononucleares de sangre periférica

RMN: Resonancia Magnética Nuclear

MS: Espectrometría de masas de alta resolución

PCA: Análisis de componentes principales (Principal Component Analaysis)

PLS-DA: Partial Least Square Discriminan Analysis

TMAO: N-óxido de trimetilamina

BCAA: aminoácidos de cadena ramificada (branched-chain amino acids)

I. RESUMEN
ABSTRACT

I. RESUMEN

Introducción

En los últimos años, la prevalencia de diabetes mellitus tipo 2 (DMT2) ha aumentado considerablemente. De hecho, se estima que el número actual de pacientes con DMT2 se duplicará en las próximas dos décadas con cifras similares para la obesidad. Por otra parte, la DMT2 puede permanecer sin diagnosticar durante muchos años porque la hiperglucemia se desarrolla de forma gradual, y, en etapas tempranas, no es lo suficientemente severa como ser detectada. Por tanto, es crucial la identificación temprana de aquellos pacientes con alto riesgo de desarrollar DMT2 para poder diseñar estrategias de prevención y controlar los factores de riesgo de la enfermedad.

Los biomarcadores tradicionales usados para identificar pacientes en riesgo de desarrollo de la enfermedad, entre los que se encuentran la glucosa plasmática en ayunas, la glucosa tras 2 horas del OGTT, hemoglobina glicada (HbA1c), parámetros relacionados con la homeostasis de glucosa, especies lipídicas y test como el Finnish Diabetes Risk Score (FINDRISC), muestran ciertas limitaciones y no son lo suficientemente eficientes y precisos. Por ello, es necesaria la búsqueda de nuevos biomarcadores que mejoren la sensibilidad, la especificidad y aumenten el valor predictivo.

La resistencia a la insulina (IR) y el deterioro de la función de las células beta son factores determinantes del desarrollo de DMT2. Estudios previos han demostrado el papel de la endotoxemia en el desarrollo de resistencia a la insulina. La absorción intestinal de componentes bacterianos, como el lipopolisacárido (LPS) de las bacterias gram negativas, activa los receptores tipo toll induciendo inflamación, y esto a su vez IR. Sin embargo, esta secuencia de eventos no está bien establecida, y aún no está claro si la IR precede a los cambios en la microbiota o viceversa. Recientemente, se ha sugerido la asociación entre la endotoxemia y el riesgo de desarrollar DMT2, no obstante, los resultados obtenidos se vieron afectados por diversos parámetros no controlados.

Adicionalmente, los microARNs (miRNAs) son reconocidos como importantes reguladores de la expresión génica y actores centrales en el control de varios procesos biológicos y patológicos, entre los que se incluyen la señalización de la insulina y la funcionalidad de la célula beta. Estudios previos sugieren el uso de los miRNAs como biomarcadores de DMT2, los cuales podrían tener una mayor sensibilidad y eficiencia para predecir el desarrollo de la enfermedad que las herramientas utilizadas actualmente. Sin embargo, son necesarios estudios longitudinales en poblaciones grandes de casos incidentes de DMT2 para conocer el papel de los miRNAs durante el desarrollo de la enfermedad y utilizar esa información en la búsqueda de nuevos biomarcadores predictivos.

Hipótesis

Nuestra hipótesis es que los niveles plasmáticos de ciertos marcadores biológicos, tales como los miRNAs, LPS y perfil de metabolitos pueden ser altamente valiosos y útiles como biomarcadores predictivos para identificar a los sujetos normoglucémicos y prediabéticos con mayor riesgo de desarrollar DMT2.

Objetivo general

Identificar nuevos biomarcadores con potencial para evaluar la probabilidad y predecir el desarrollo DMT2 en pacientes con enfermedad coronaria de alto riesgo.

Sujetos, diseño y metodología de las publicaciones

Artículo 1: En este trabajo se incluyeron todos los pacientes no diagnosticados con diabetes al inicio del estudio CORDIOPREV (NTC00924937). De ellos, 107 pacientes desarrollaron DMT2 de acuerdo con los criterios de diagnóstico de la Sociedad Americada de Diabetes (ADA) tras una mediana de seguimiento de 60 meses (grupo Incident-DIAB). Se llevó a cabo una sobrecarga grasa y una sobrecarga oral de glucosa (OGTT) en días consecutivos al inicio del estudio y tras 3 años de seguimiento. Seguidamente, se determinaron los niveles plasmáticos de LPS, LBP (proteína de unión a lipopolisacárido), IL-6, MCP1 y TNF-alpha. Por último, se realizaron análisis de regresión de COX para evaluar el potencial de los niveles postprandiales de LPS como predictor del desarrollo de DMT2.

Artículo 2: Este trabajo se llevó a cabo en la misma población no diabética al inicio del estudio CORDIOPREV. Los niveles plasmáticos de 24 miRNAs se determinaron en la situación basal mediante qRT-PCR. Además se realizaron análisis de componentes principales (OPLS-DA), curvas ROC y de regresión de COX para evaluar el poder predictivo de los miRNAs circulantes en el desarrollo de DMT2.

Artículo 3: Se incluyeron los 462 pacientes sin diabetes al inicio del estudio CORDIOPREV. Tras una mediana de seguimiento de 60 meses, 107 de ellos desarrollaron DMT2, 30 desarrollaron prediabetes, 223 mantuvieron la prediabetes y 78 estaban libres de enfermedad. Los niveles plasmáticos de cuatro miRNAs relacionados con la señalización de la insulina y la funcionalidad de la célula beta se determinaron mediante qRT-PCR. Adicionalmente, analizamos la relación entre los niveles de miRNAs y los índices relacionados con la señalización de la insulina en la situación basal y después del periodo de seguimiento. El riesgo de desarrollar la enfermedad fue evaluado mediante análisis de regresión de COX en función de los niveles de miRNAs en la situación basal categorizados por tertiles.

Otra aproximación: Seleccionamos un grupo de 52 pacientes sin diabetes al inicio del estudio CORDIOPREV, 26 de los cuales desarrollaron la enfermedad tras 3 años de seguimiento y otros 26 que no la desarrollaron. Estudiamos las diferencias en el perfil metabolómico entre ambos grupos en muestras de plasma tomadas en la situación basal, tanto en el estado de ayuno como en el postprandial. Se llevaron a cabo análisis de componentes principales (PLS-DA y OPLS-DA) y de curvas ROC con los niveles plasmáticos de los metabolites identificados para evaluar el poder predictivo del desarrollo de DMT2.

Resultados

Artículo 1: Observamos un aumento en los niveles de LPS postprandiales en la situación basal en los pacientes que desarrollan diabetes ($P < 0.001$), mientras que no se encontraron cambios en el grupo de no-diabéticos. El análisis de regresión de COX, basado en los cambios postprandiales de LPS, mejoró el poder predictivo del desarrollo de DMT2 en comparación con el FINDRISC (hazard ratio de 2.076, 95% CI 1.149-3.750 vs. 1.384, 95% CI 0.740-2.589). Además, el análisis de regresión de COX combinando ambos parámetros mostró que los pacientes con niveles postprandiales elevados de LPS y alto FINDRISC tienen un mayor riesgo de desarrollo de diabetes (hazard ratio de 3.835 (95% CI 1.323-11.114)).

Artículo 2: El análisis de ROC identificó 9 miRNAs, los cuales, añadidos a la HbA1c, tienen un mayor valor predictico en el diagnóstico temprano de la DMT2 (AUC = 0.8342), en comparación con la HbA1c (AUC = 0.6950). Este modelo de miRNAs y HbA1c no mejoró cuando se incluyó el FINDRISC (AUC = 0.8293). El análisis de regresión de COX mostró que los pacientes con bajos niveles circulantes de *miR-103*, *miR-28-3p*, *miR-29a*, y *miR-9,* y, altos de *miR-30a-5p* y *miR-150* en la situación basal tienen mayor riesgo de desarrollar la enfermedad (HR = 11.27; 95% CI = 2.61–48.65).

Artículo 3: Observamos mayores niveles de *miR-150* y *miR-30a-5p* y menores niveles de *miR-15a* y *miR-375* en la situacion basal en los pacientes que desarrollan DMT2 que en los que no la desarrollan. Además, los pacientes con niveles altos de *miR-150* y *miR-30a-5p* mostraron una peor funcionalidad de la célula beta (menor disposition index) (p=0.047 y p= 0.007, respectivamente). Por último, un mayor riesgo de desarrollar DMT2 también se asoció con altos niveles (T3) de *miR-150* y *miR-30a-5p* (HR_{T3-T1} = 4.218 y HR_{T3-T1} = 2.527, respectivamente), y bajos niveles (T1) de *miR-15a* y *miR-375* (HR_{T1-T3} = 3.269 and HR_{T1-T3} = 1.604, respectivamente) en la situación basal.

Otra aproximación: Nuestro estudio sugiere que el perfil de metabolitos en la situación basal, tanto en el estado de ayunas como en el postprandio, permite diferenciar entre sujetos que desarrollan DMT2 y aquellos que no la desarrollan tras tres años de seguimiento. De hecho, el análisis de curvas ROC mostró que los niveles plasmáticos de metabolitos específicos podrían tener potencial como biomarcadores predictivos del desarrollo de la enfermedad. Nuestros resultados mostraron un AUC de 0.794 (sensibilidad = 0,808, especificidad= 0.731 y precisión= 72.8%) en un modelo construido con los metabolitos identificados en el estado de ayunas y con VIP>1.5, lo cuales pertenecen principalmente a la famila de las glicerofosfocholinas. Además, obtuvimos un AUC de 0.849 (sensibilidad = 0,814, especificidad= 0.727 y precisión= 74.1%) con los metabolitos identificados en el análisis postprandial, los cuales pertenecen en su mayoría a la familia de ácidos grasos.

Conclusión

Los niveles plasmáticos de ciertos miRNAs, LPS y perfil de metabolitos podrían tener potencial como biomarcadores para evaluar la probabilidad y predecir el desarrollo DMT2 en pacientes de alto riesgo con enfermedad coronaria.

I. ABSTRACT

Introduction

During the last years, the prevalence of type 2 diabetes mellitus (T2DM) has steadily increased; it has been estimated that the current number of T2DM patients worldwide will double over the next two decades, and similar trends have been reported for obesity. In addition, T2DM frequently goes undiagnosed for many years because hyperglycemia develops gradually, and, in its earlier stages, is not severe enough for the patient to note any of the classic symptoms of diabetes. It is, therefore, critical that there be early identification of individuals at high risk for developing diabetes in order to devise prevention strategies aimed at controlling the factors related to the development of the disease.

The traditional biomarkers used to identify patients at risk of developing the disease, including fasting glucose, glucose after 2 hours of OGTT, HbA1c, glucose-homeostasis-related parameters, lipid types and scores such as the Finnish Diabetes Risk Score (FINDRISC), have limitations leading to low accuracy and efficiency. Therefore, it is necessary to research into new biomarkers to improve sensitivity and specificity and increase the predictive value.

Insulin resistance (IR) and impaired beta-cell function are key determinants of type 2 diabetes mellitus (T2DM). Previous studies have demonstrated the role of endotoxemia in the develop of IR. Intestinal absorption of bacterial components such us endotoxin lipopolysaccharides (LPS) activates the toll-like receptors inducing inflammation, and this in turn, leads to IR. However, this sequence of events is not well-established, and it is still unclear whether IR precedes the changes in the microbiota or vice-versa. Recently, an association between endotoxemia and the risk of developing diabetes was demonstrated; although, this study was subject to severe, uncontrolled confounding factors.

In addition, microRNAs (miRNAs) are recognized as important regulators of gene expression and central players in the control of several biological and pathological processes, including insulin signaling and beta cell dysfunction. Previous studies suggest the use of miRNAs as biomarkers of T2DM, and they may be a more sensitive way to predict development of the disease than the tools currently used. However, long-term follow-up studies in larger populations with incident cases of T2DM are necessary

to identify the role of miRNAs during the development of the disease and to use that information to help identify new T2DM predictive biomarkers.

Hypothesis

Our hypothesis is that plasma levels of different biological markers, such as miRNAs, LPS and metabolite profile, could become highly valuable and useful as predictive biomarkers to identify normoglycemic and prediabetic patients with a higher risk of developing T2DM.,

Objectives

To identify new biomarkers with the potential to evaluate the probability and predict the develop of type 2 diabetes mellitus in high-risk patients with coronary heart disease.

Subjects, design and methodology of the publications

Paper 1: We included in the present work the 462 patients who had not been clinically diagnosed with T2DM at baseline in the CORonary Diet Intervention with Olive oil and cardiovascular PREVention study (CORDIOPREV)(NTC00924937). Of these, 107 patients developed T2DM according to the American Diabetes Association (ADA) diagnosis criteria after a median follow-up of 60 months (Incident-DIAB group), whereas 355 patients did not develop the disease during this period (Non-DIAB group). Two metabolic challenges, a fat overload and an oral glucose tolerance test, were performed on consecutive days at the beginning of the study and after 3 years of follow-up. Plasma levels of LPS were measured in the whole population and lipopolysaccharide binding protein (LBP), IL-6, MCP1 and TNF-a were determined in a subpopulation of 226 patients. Cox proportional hazards regression analyses were performed in order to determine the potential use of LPS postprandial levels as a predictor of T2DM development.

Paper 2: The study was conducted in the 462 non-diabetic patients at baseline of the CORDIOPREV study. The plasma levels of 24 miRNAs were measured at baseline by qRT-PCR, and other strong biomarkers to predict diabetes were also measured. Orthogonal Partial Least Squares Discriminant (OPLS-DA), Receiver Operating Characteristic (ROC) and Cox regression analysis were used to evaluate the potential predictive value of the circulating miRNAs in the development of T2DM.

Paper 3: This study included the 462 patients without disease at baseline from the CORDIOPREV study. After a median follow-up of 60 months, 107 of the subjects developed the disease, 30 developed prediabetes, 223 maintained prediabetes and 78 remained disease-free. The plasma levels of four miRNAs linked to insulin signaling and beta-cell function were measured by RT-PCR. We analyzed the relationship between the miRNAs levels and insulin signaling and release indexes at baseline and after the follow-up period. The risk of developing the disease was evaluated through a Cox analysis based on tertiles (T1-T2-T3) of baseline miRNAs levels.

Other approach: A group of 52 patients from the CORDIOPREV study without T2DM at baseline was selected, 26 of whom developed the disease after 3 years of follow-up. We studied the differences in the metabolomic profile between groups in plasma samples in the fasting and postprandial states at baseline. Principal component analysis (PLS-DA and OPLS-DA) and ROC analysis were carried out with the plasma levels of the metabolites identified to evaluate the predictive value of T2DM development.

Results

Paper 1: We observed a postprandial increase in lipopolysaccharide (LPS) levels in the Incident-DIAB at baseline ($P < 0.001$), whereas LPS levels were not modified in the Non-DIAB. The COX regression analysis based on the LPS postprandial fold change improved the T2DM risk assessment as compared with the previously described FINDRISC score (hazard ratio of 2.076, 95% CI 1.149-3.750 vs. 1.384, 95% CI 0.740-2.589). Moreover, a COX regression analysis merging the LPS postprandial fold change and FINDRISC score together showed a hazard ratio of 3.835 (95% CI 1.323-11.114), linked to high values of both parameters.

Paper 2: The ROC analysis identified 9 miRNAs, which, added to HbA1c, have a greater predictive value in the early diagnosis of type 2 diabetes (AUC = 0.8342) than HbA1c alone (AUC = 0.6950). The miRNA and HbA1c-based model did not improve when the FINDRISC was included (AUC = 0.8293). The COX regression analyses showed that patients with low *miR-103, miR-28-3p, miR-29a, and miR-9* and high *miR-30a-5p* and *miR-150* circulating levels have a higher risk of disease (HR = 11.27; 95% CI = 2.61–48.65).

Paper 3: We observed higher *miR-150* and *miR-30a-5p* and lower *miR-15a* and *miR-375* baseline levels in type 2 diabetes than disease-free subjects. Patients with high *miR-150* and *miR-30a-5p* baseline levels had lower disposition index (p=0.047 and p= 0.007, respectively). A higher risk of disease was associated with high levels (T3) of *miR-150* and *miR-30a-5p* ($HR_{T3\text{-}T1}$ = 4.218 and $HR_{T3\text{-}T1}$ = 2.527, respectively), and low levels (T1) of *miR-15a* and *miR-375* ($HR_{T1\text{-}T3}$ = 3.269 and $HR_{T1\text{-}T3}$ = 1.604, respectively).

Other approach: Our study suggests that in both the fasting and postprandial state, the metabolomic profile allows us to differentiate between subjects who develop (new-T2DM) or do not develop (non-T2DM) type 2 diabetes mellitus after 3 years of follow-up. In fact, the ROC analysis showed that the plasma levels of specific metabolites could potentially be used as biomarkers to predict the development of the disease. Our results show an AUC of 0.794 (sensibility = 0,808, specificity= 0.731 and accuracy= 72.8%) in a model designed with the metabolites identified in the fasting analysis and a VIP score>1.5, which belongs mostly to the glycerophosphocholines family. In addition, we obtained an AUC of 0.849 (sensibility = 0,814, specificity= 0.727 and accuracy= 74.1%) with the metabolites identified in the postprandial analysis, which belong mostly to the fatty acids family.

Conclusion

The plasma levels of certain miRNAs, LPS and metabolite profile could have a valuable potential as biomarkers to evaluate the probability and predict the develop of type 2 diabetes mellitus in high-risk patients with coronary heart disease.

II. INTRODUCTION

II. INTRODUCTION

1. DIABETES MELLITUS

1.1 Antecedentes Históricos

La diabetes es una enfermedad con un origen muy antiguo, que se remonta al año 1550 A.C, cuando un papiro egipcio mencionaba una rara enfermedad que causaba en los pacientes una rápida pérdida de peso y una alta frecuencia urinaria. El término “diabetes” se le atribuye a Apollonius de Memphis, refiriendose a una enfermedad en la que los pacientes drenan más líquido del que consumen. En el siglo XI, la uroscopia se convierte en una forma de diagnóstico de la diabetes, así mediante el examen del color, el sedimento y el olor de la orina se pretendía establecer las causas de la enfermedad en el paciente. Algunos médicos incluso probaron la orina, y aparentemente es así como a la diabetes se le da su segundo nombre, mellitus, que significa "miel" en latín. Más tarde, entre los siglos XVIII y XIX, Matthew Dobson demuestra que el sabor dulce en la orina de las personas con diabetes se debe al exceso de glucosa tanto en orina como en sangre. En 1910, el estudio del páncreas por parte del fisiólogo inglés Sir Edward Albert Sharpey-Schafer lo llevó al descubrimiento de la insulina. El nombre proviene del latin “insula”, que significa isla, haciendo referencia a los islotes pancreáticos de Langerhans donde se sintetiza la insulina. En 1940, se fundó la American Diabetes Association (ADA) con el objetivo de estudiar la creciente incidencia de diabetes y las complicaciones que se derivan de la enfermedad. Posteriormente, en los años 60, utilizando la tecnología de radioinmunoanálisis, Solomon Berson, MD y Rosalyn Yalow, PhD desarrollaron un método para determinar los niveles de insulina en sangre. Ellos observaron que algunas personas con diabetes siguen produciendo su propia insulina, lo que les permitió clasificar a los pacientes como "insulinodependientes" (tipo 1) y "no insulinodependientes" (tipo 2). En 1977, se desarrolló la prueba para medir la hemoglobina glicosilada (HbA1c), que años más tarde se convierte en el “goldem standard” para el diagnóstico de diabetes y prediabetes. A mediados de los años 90, se descubrió la hormona incretina GLP-1 (péptido similar al glucagón tipo 1) y el medicamento metformina empezó a estar disponible en EE. UU. Desde sus orígenes hasta la actualidad, la diabetes mellitus se ha diagnosticado en millones de personas ocasionando un gran número de muertes y, dadas sus complicaciones, disminuyendo la calidad de vida de quienes la padecen [1].

1.2 Aspectos clínicos y clasificación

La diabetes mellitus la forman un grupo de enfermedades metabólicas caracterizadas por hiperglucemia, como resultado de defectos en la secreción de insulina, la acción de la insulina o ambas. La hiperglucemia crónica en la diabetes se asocia con daño a largo plazo, disfunción y alteraciones en varios órganos, especialmente los ojos, los riñones, el corazón y los vasos sanguíneos [2]. Diversos procesos patogénicos están implicados en el desarrollo de diabetes, que van desde la destrucción autoinmune de las células beta del páncreas con la consecuente deficiencia en la secreción de insulina hasta anormalidades que resultan en resistencia a la insulina. La deficiente actividad de la insulina en los tejidos diana es la base de las alteraciones en el metabolismo de carbohidratos, grasas y proteínas que subyacen a la diabetes. El deterioro en la secreción y actividad de la insulina coexisten con frecuencia en el mismo paciente, sin embargo, de estas dos alteraciones no está claro cuál es la causa principal de la hiperglucemia. Dentro de los síntomas más comunes asociados a la hiperglucemia se incluyen la poliuria, polidipsia, pérdida de peso y, a veces, polifagia, visión borrosa y susceptibilidad a ciertas infecciones. Las complicaciones a largo plazo de la diabetes incluyen retinopatía con pérdida potencial de la visión; nefropatía que conduce a insuficiencia renal; neuropatía periférica con riesgo de úlceras en el pie, amputaciones y articulaciones de Charcot; y neuropatía autonómica que causa síntomas gastrointestinales, genitourinarios, cardiovasculares y disfunción sexual. Los pacientes con diabetes tienen un mayor riesgo de desarrollar enfermedad cardiovascular, enfermedad arterial periférica y enfermedad cerebrovascular aterosclerótica. En la actualidad, la diabetes mellitus se clasifica según las causas por las cuales se alcanza el estado de hiperglucemia, así la gran mayoría de los casos de diabetes se clasifican en dos grandes categorías etiopatogénicas: diabetes tipo 1 y diabetes tipo 2. En la diabetes tipo 1 hay una deficiencia absoluta de secreción de insulina. Las personas con mayor riesgo de desarrollar este tipo de diabetes a menudo pueden ser identificadas por evidencia serológica de un proceso patológico autoinmune que ocurre en los islotes pancreáticos y por marcadores genéticos. En la otra categoría, mucho más prevalente, la diabetes mellitus tipo 2, la causa es una combinación de resistencia a la insulina y una inadecuada respuesta compensatoria de secreción de insulina. Diagnosticar un tipo de diabetes u otro a menudo depende de las circunstancias presentes en el momento del diagnóstico, y muchas personas diabéticas no encajan fácilmente en una sola clase [3].

1.2.1. Diabetes mellitus tipo 1

La diabetes mellitus tipo 1 (DMT1) se caracteriza por la destrucción de las células beta en un proceso mediado por el sistema inmunitario, lo que generalmente conlleva a una deficiencia absoluta de insulina. Conocida anteriormente como "diabetes insulinodependiente o diabetes juvenil", representa solo el 5-10% de los casos de diabetes. Los marcadores de la destrucción inmune de la célula beta incluyen autoanticuerpos de las células de los islotes, autoanticuerpos contra insulina, autoanticuerpos contra la enzima glutamato decarboxilasa (GAD) y contra las enzimas tirosina fosfatasas IA-2 y IA-2b. Al menos uno y generalmente más de uno de estos anticuerpos están presente en el 85-90% de los casos.

Además, la enfermedad tiene un fuerte componente genético, se desregulan genes del complejo mayor de histocompatibilidad, incluyendo a los genes que codifican al antígeno leucocitario humano (human leucocyte antigen, HLA), crucial para la presentación de antígenos. La región del HLA, situada en el cromosoma 6p21,3, una región de 3,5 Mb con >200 genes, es la región más establecida de riesgo de DMT1. El HLA I contiene HLA-A, HLA-B y HLA-C; el HLA II contiene HLA-DR (DRA, DRB), HLA-DQ (DQA, DQB) y HLA-DP (DPA, DPB), y el HLA III, localizado entre el I y el II, incluye a los genes del complemento C2, C4, Bf, genes de proteínas de choque térmico (HSP70), genes del factor de necrosis tumoral (TNF), 21-hidroxilasa (21-OH) y muchos más [4]. En esta forma de diabetes, la tasa de destrucción de células beta es bastante variable, siendo rápida en algunos individuos (principalmente bebés y niños) y lenta en otros (principalmente adultos). Algunos pacientes, particularmente niños y adolescentes, pueden presentar cetoacidosis como la primera manifestación de la enfermedad. Otros pacientes muestran una leve hiperglucemia en ayunas que puede cambiar rápidamente a hiperglucemia grave y / o cetoacidosis en presencia de infección u otro tipo de estrés. Algunos pacientes, particularmente adultos, pueden mantener la suficiente acción residual de la célula beta como para prevenir la cetoacidosis durante muchos años [5].

En las etapas más avanzadas de la enfermedad, hay poca o ninguna secreción de insulina, manifiesta por los bajos o indetectables niveles de péptido C en plasma. La diabetes mediada por inmunidad ocurre comúnmente en la niñez y la adolescencia, pero puede ocurrir a cualquier edad, incluso hasta los 80 años. La destrucción autoinmune de las células beta, aunque con una amplia predisposición genética, también está relacionada con diversos factores ambientales que aún están poco definidos [3]. Aunque los pacientes con DMT1 rara vez son obesos, la presencia de obesidad no es incompatible con el diagnóstico. Estos pacientes también son propensos a otros trastornos autoinmunes, como Enfermedad de Graves, tiroiditis de Hashimoto, enfermedad de Addison, vitíligo, esprue celíaco, hepatitis autoinmune, miastenia gravis y anemia perniciosa [6].

1.3 Diabetes mellitus tipo 2

Esta forma de diabetes, que representa entre el 90 y el 95% de los casos, fue conocida previamente como diabetes no insulinodependiente o diabetes de inicio en adultos e incluye pacientes que muestran resistencia a la insulina y/o con una relativa deficiencia en la síntesis de insulina. Al menos inicialmente, y a menudo durante toda su vida, estas personas no necesitan tratamiento con insulina para sobrevivir.

Estudios previos demuestran que existen diversas causas asociadas al desarrollo de esta forma de diabetes, sin embargo, la principal es la obesidad asociada a la resistencia a la insulina. La resistencia a la insulina puede mejorar con la perdida de peso y / o el tratamiento farmacológico para la hiperglucemia, pero rara vez se restablece la normalidad [3]. La diabetes tipo 2 a menudo permanece sin diagnosticar durante varios años, ya que la hiperglucemia se desarrolla gradualmente y, en etapas más tempranas a menudo no es lo suficientemente grave como para que el paciente note ninguno de los síntomas clásicos de la enfermedad. Sin embargo, tales pacientes tienen un mayor riesgo de desarrollar complicaciones macrovasculares y microvasculares [7].

Ocurre con mayor frecuencia en mujeres con diabetes mellitus gestacional (DMG) previa y en individuos con hipertensión o dislipidemia, y su frecuencia varía en diferentes subgrupos raciales / étnicos. A menudo la diabetes mellitus tipo 2 se asocia con una fuerte predisposición genética, más que la DMT1, sin embargo, la genética de esta forma de diabetes es más compleja y no está completamente definida [8, 9].

1.3.1. Aspectos clínicos de la diabetes mellitus tipo 2

Como se ha mencionado previamente, la diabetes mellitus se caracteriza por la alteración en la homeostasis de la glucosa, estado que conlleva al aumento de los niveles de glucosa en sangre, debido a la baja producción / secreción de insulina por las células beta pancreáticas y a una baja respuesta por parte de los tejidos diana (músculo esquelético, tejido adiposo, hígado etc.). La hiperglucemia ocasiona cambios patológicos y funcionales en varios tejidos diana, sin embargo, la manifestación de estas alteraciones puede estar ausente durante un largo período de tiempo antes de que la enfermedad se diagnostique. Durante este período asintomático, existe una alteración en el metabolismo de los carbohidratos, que puede llegar a detectarse mediante la determinación de la concentración plasmática de glucosa en ayunas o tras una sobrecarga oral de glucosa. Niveles elevados de glucosa en cualquiera de los dos momentos sugieren hiperglucemia, IFG (en inglés impaired fasting glucose – alteración en los niveles de glucosa en ayunas) e IGT (en inglés impaired glucose tolerance – alteración en la glucosa tras 2 horas de una sobrecarga oral). Sin embargo, puede que no lo suficiente como para generar un imbalance en los niveles de glucosa y cumplir los criterios de diagnóstico de diabetes. En algunas personas con diabetes, se puede lograr un control glucémico adecuado gracias a la adopción de hábitos saludables como el ejercicio, la subsecuente pérdida de peso, y / o por la acción de fármacos como los agentes hipoglucemiantes orales [7], sin requerir la utilización de insulina. Por otra parte, existen algunos pacientes que tienen cierta capacidad de secreción de insulina residual pero requieren de la utilización de insulina exógena para el control glucémico adecuado para sobrevivir. La gravedad de la anomalía metabólica puede progresar, retroceder o permanecer igual. Por lo tanto, el grado de hiperglucemia refleja la gravedad del proceso metabólico subyacente y permite definir la terapia a seguir para el control de la enfermedad [10].

Los síntomas de la DMT2 que pueden observarse en el momento del diagnóstico son sed, poliuria, fatiga y malestar general, infecciones (especialmente candidiasis genital) y visión borrosa. Estos síntomas, que generalmente se observan en personas mayores, no suelen ser de naturaleza tan grave o de inicio tan repentino como los observados en la DMT1 (o insulinodependiente). Es importante reconocer que todos los pacientes diabéticos corren el riesgo de desarrollar complicaciones. La mayor parte de la morbilidad y mortalidad asociadas con la diabetes mellitus tipo 2 se le atribuye a las complicaciones crónicas de la enfermedad, las cuales pueden ser de tipo microvascular o macrovascular [11].

Las principales complicaciones microvasculares de la diabetes mellitus son las siguientes:

Retinopatía diabética

En los países occidentales, la diabetes mellitus es la causa más común de ceguera en personas de entre 20 y 60 años. Alrededor del 5% al 10 % de los pacientes con diabetes pueden tener retinopatía en el momento del diagnóstico. En los pacientes con diabetes mellitus tipo 2, la perdida de la visión es ocasionada porque los niveles altos de glucosa en sangre causan daño a los vasos sanguíneos de la retina, los cuales pueden hincharse y tener fugas de líquido. También pueden cerrarse e impedir que la sangre fluya. También, a veces, se generan nuevos vasos sanguíneos anormales en la retina. Todos estos cambios inducen pérdida de la visión [11].

Nefropatía diabética

La enfermedad renal diabética afecta al 15-20% de los sujetos con diabetes mellitus tipo 2, el 50% de los cuales progresará a insuficiencia renal. La nefropatía diabética o enfermedad renal diabética es un síndrome caracterizado por la presencia de cantidades patológicas de excreción de albúmina en la orina, lesiones glomerulares diabéticas y una disminución en la tasa de filtración glomerular (TFG) [12]. La nefropatía diabética se está convirtiendo en la causa más común de insuficiencia renal en etapa terminal. En Europa, más del 10% de los pacientes que requieren diálisis o trasplante tienen diabetes mellitus y esta cifra es dos veces más alta en los EE. UU [11].

Neuropatía diabética

La forma más común de daño a los nervios es la neuropatía periférica crónica en la que se pierde la apreciación del tacto y el dolor, seguida de la pérdida de la propiocepción en las extremidades inferiores. Los pies están entumecidos, se dañan fácilmente por el calor o pequeños traumatismos y pueden ocurrir ulceraciones. La neuropatía periférica diabética es difícil de tratar y el aspecto más importante sobre el que hacer incapié es la educación del paciente en los principios básicos del buen cuidado del pie [13].

Pie diabético

Los problemas en los pies son una causa importante de morbilidad en la DMT2 y uno de los motivos más frecuentes de ingresos hospitalarios. El pie diabético representa un conjunto de trastornos que incluyen desde insuficiencia vascular, neuropatía e infección hasta gangrena. La neurotopatía sensorial crónica (pies entumecidos) es un factor importante en el 70-80% de las úlceras del pie diabético y suele ser una característica permanente. El cuidado preventivo de los pies es esencial en pacientes con DMT2. La educación del paciente en los principios básicos del buen cuidado del pie es esencial y todos los pacientes deben ser vistos regularmente por un podólogo [11].

Por otro lado, las complicaciones macrovasculares no son exclusivas de la diabetes, sino que ocurren mucho más frecuentemente en sujetos diabéticos. Dentro de las complicaciones macrovasculares se incluyen:

Enfermedad cardiovascular

Esta es la principal causa de muerte y hospitalización en sujetos con DMT2. El infarto agudo de miocardio es de dos a cuatro veces más frecuente en pacientes con DMT2 que en la población general y la mortalidad por insuficiencia cardíaca y ataques cardíacos también es más alta. La edad, el control de la glucemia, el aumento de la presión arterial sistólica, niveles elevados de colesterol LDL con niveles reducidos de colesterol HDL y el tabaquismo son factores de riesgo significativos de enfermedad cardiovascular en pacientes con diabetes mellitus tipo 2 [14, 15].

Enfermedad cerebrovascular y Enfermedad vascular periférica

El accidente cerebrovascular isquémico agudo y los ataques isquémicos cerebrales transitorios son de tres a cuatro veces más comunes en la DMT2. La incidencia de enfermedad vascular periférica se incrementa hasta seis veces, pudiendo presentarse como claudicación intermitente, ulceración o gangrena. La amputación de miembros inferiores es hasta 20 veces más común en sujetos con diabetes mellitus tipo 2 [16, 17].

1.3.2 Parámetros y criterios de diagnóstico de la diabetes mellitus tipo 2

La DMT2 puede diagnosticarse según los criterios de glucosa plasmática, ya sea la glucosa plasmática en ayunas (**FPG**) la glucosa plasmática a las 2 h (**2h-PG**) tras un test de sobrecarga oral de glucosa (OGTT) de 75 g [18] o niveles elevados de hemoglobina glicosilada en sangre (HbA1c)(**Tabla 1**) [3, 19]. La eficacia de las intervenciones para la prevención primaria de la diabetes tipo 2 [20] se ha demostrado principalmente entre personas con intolerancia a la glucosa (IGT), no para personas con glucosa alterada aislada en ayunas (IFG) o para aquellos con prediabetes definidos por los criterios de HbA1c. Las mismas pruebas se pueden usar para detectar y diagnosticar diabetes como para identificar personas con prediabetes.

Glucosa plasmática en ayunas y 2 horas

La glucosa plasmática en ayunas (FPG) y la glucosa plasmática a las 2 h (2h PG) se usan para diagnosticar diabetes (**Tabla 1**). La concordancia entre las pruebas de FPG y 2h-PG es imperfecta, como lo es la concordancia entre HbA1c y cualquiera de las pruebas basadas en glucosa.

Hemoglobina glicosilada (HbA1c)

La HbA1c tiene varias ventajas en comparación con FPG y 2h-PG, entre las que se incluyen una mayor comodidad (no se requiere ayuno), una mayor estabilidad preanalítica y menos perturbaciones diarias durante el estrés y la enfermedad. Sin embargo, estas ventajas pueden ser compensadas por la menor sensibilidad de la HbA1c en el punto de corte designado, mayor costo, disponibilidad limitada de pruebas de HbA1c en ciertas regiones del mundo en desarrollo, y la correlación imperfecta entre HbA1c y glucosa promedio en ciertos individuos. Los datos de la Encuesta Nacional de Salud y Nutrición (NHANES) indican que un punto de corte de HbA1c ≥ 6.5% (48 mmol / mol) identifica un tercio menos de casos de diabetes no diagnosticada que un punto de corte de glucosa en ayuno de 126 mg / dL (7.0 mmol / L) [21]. Cuando se usa HbA1c para diagnosticar diabetes, es importante reconocer que HbA1c es una medida indirecta de los niveles promedio de glucosa en sangre y tomar en consideración otros factores que pueden afectar la glicación de la hemoglobina independientemente de la glucemia, incluida la edad, raza / etnia y anemia / hemoglobinopatías [3].

Confirmación del diagnóstico

A menos que exista un diagnóstico clínico claro (por ejemplo, paciente en crisis hiperglucémica o con síntomas clásicos de hiperglucemia y una glucosa plasmática aleatoria ≥ 200 mg / dL [11.1 mmol / L]), se requiere una segunda prueba para la confirmación. Se recomienda que la misma prueba se repita sin demora utilizando una nueva muestra de sangre. Por otro lado, si un paciente tiene resultados discordantes de dos pruebas diferentes, se debe repetir el resultado de la prueba que está por encima del punto de corte de diagnóstico. El diagnóstico se realiza teniendo en cuenta la prueba confirmada. Por ejemplo, si un paciente cumple con el criterio de diabetes de la HbA1c (dos resultados ≥ 6,5% [48 mmol / mol]) pero no FPG (<126 mg / dL [7,0 mmol / L]), esa persona debe considerarse diabética. Sin embargo, dado que todas las pruebas tienen variabilidad preanalítica y analítica, es posible que un resultado anormal (es decir, por encima del umbral de diagnóstico), cuando se repita, produzca un valor por debajo del punto de corte de diagnóstico. Este escenario es probable para FPG y 2-h PG si las muestras de glucosa permanecen a temperatura ambiente y no se centrifugan rápidamente. Debido al potencial de variabilidad preanalítica, es fundamental que el plasma se centrifugue y se separe inmediatamente después de la extracción. Si los pacientes tienen resultados cerca de los márgenes del umbral de diagnóstico, el profesional de la salud debe seguir de cerca al paciente y repetir la prueba en 3-6 meses [7].

Tabla 1. Criterios de diagnostico de diabetes mellitus tipo 2 según la Sociedad Americana de Diabetes (ADA) [22]

HbA1c ≥ 6.5% (48 mmol/mol)
ó
FPG≥126 mg/dl (7.0 mmol/l)
ó
Glucosa plasmática tras 2-h del OGTT ≥ 200 mg/dl (11.1mmol/l) El test debe realizarse según lo establecido por la Sociedad Americana de Diabetes (ADA), con una sobrecarga de 75 g de glucose anhidra disuelta en agua.
ó
Paciente con síntomas clásicos de hiperglucemia o crisis hiperglucémica o glucosa plasmática al azar ≥ 200 mg/dl (11.1 mmol/l)

*En ausencia de hiperglucemia clara, los criterios 1-3 deberían ser confirmados repitiendo el diagnóstico

Categorías con riesgo aumentado de diabetes (prediabetes)

En 1997 y 2003, el Comité de Expertos sobre el Diagnóstico y Clasificación de la Diabetes Mellitus [23, 24] reconoció que había un grupo de individuos cuyos niveles de glucosa no cumplían con los criterios de diabetes pero eran demasiado altos para ser considerados normales. "Prediabetes" es el término utilizado para individuos con IFG y / o IGT y / o HbA1c entre 5.7-6.4% (39-47 mmol / mol). La prediabetes no debe considerarse como una entidad clínica en sí misma, sino más bien como un mayor riesgo de diabetes (Tabla 2) y enfermedad cardiovascular. La prediabetes se asocia con obesidad (especialmente obesidad abdominal o visceral), dislipidemia con niveles altos de triglicéridos y / o bajos de colesterol HDL e hipertensión. El Comité de Expertos para el Diagnóstico y Clasificación de la Diabetes Mellitus [23, 24] definió IFG como niveles de FPG entre 100 y 125 mg / dL (entre 5,6 y 6,9 mmol / L)) e IGT como 2h-PG entre 140 y 199 mg / dL (entre 7,8 y 11,0 mmol / l). Cabe señalar que la Organización Mundial de la Salud (OMS) y muchas otras organizaciones de diabetes definen el límite de IFG en 110 mg / dL (6.1 mmol / L).

En una revisión sistemática de 44.203 individuos pertenecientes a 16 cohortes con un intervalo de seguimiento de 5.6 años (rango 2.8-12 años), aquellos con HbA1c entre 5.5 y 6.0% (entre 37 y 42 mmol / mol) tuvieron un riesgo sustancialmente mayor de diabetes (incidencia a 5 años del 9 al 25%). Un rango de HbA1c de 6.0-6.5% (42-48 mmol / mol) tuvo un riesgo entre 25 y 50% de desarrollar diabetes en 5 años y un riesgo relativo 20 veces mayor en comparación con niveles de HbA1c del 5.0% (31 mmol / mol)[25]. En un estudio basado en una comunidad de adultos blancos afroamericanos y no hispanos sin diabetes, la HbA1c fue un predictor más fuerte de diabetes y eventos cardiovasculares posteriores que la glucosa en ayunas [26]. Otros análisis sugieren que una HbA1c de 5.7% (39 mmol / mol) o más se asocia con un riesgo de diabetes similar al de los participantes de alto riesgo en el Programa de Prevención de la Diabetes (DPP) [27], donde la HbA1c fue un fuerte predictor del desarrollo de diabetes [28].

Por lo tanto, es razonable considerar un rango de HbA1c de 5.7-6.4% (39-47 mmol / mol) para identificar a las personas con prediabetes. De forma similar a aquellas personas con IFG y / o IGT y HbA1c de 5.7-6.4% (39-47 mmol / mol) deben ser informadas de su mayor riesgo de desarrollar diabetes y ECV, así como aconsejarles sobre estrategias preventivas. Similar a las mediciones de glucosa, el riesgo de desarrollar la enfermedad aumenta al aumentar los niveles de HbA1c [25]. Se deben buscar intervenciones eficaces y realizar un seguimiento de aquellas personas consideradas de muy alto riesgo (p. Ej., aquellos con HbA1c .6.0% [42 mmol / mol]). La **tabla 2** resume los criterios de prediabetes y la **tabla 3** los criterios para seleccionar a los adultos asintomáticos en los que realizar las pruebas de prediabestes o diabetes.

Tabla 2- Criterios de prediabetes según la ADA [22]

FPG 100 mg/dL (5.6mmol/L) a 125 mg/dL (6.9 mmol/L) (IFG)
ó
Glucosa plasmática tras 2 horas del OGTT entre 140 mg/dL (7.8 mmol/L) y 199 mg/dL (11.0 mmol/L) (IGT)
ó
HbA1c entre 5.7–6.4%

Tabla 3-Criterios para seleccionar a los adultos asintomáticos en los que testar el diagnóstico de prediabetes o diabetes

1. Las pruebas deben considerarse en adultos con sobrepeso u obesidad (IMC ≥ 25 kg / m2 o ≥ 23 kg / m2 en asiático-americanos) con uno o más de los siguientes factores de riesgo:
• HbA1c ≥ 5.7% (39 mmol / mol), IGT o IFG en pruebas previas • antecedente familiar de primer grado con diabetes • raza / etnia de alto riesgo (por ejemplo, afroamericano, latino, nativo americano, asiático-americano, isleño del Pacífico) • mujeres que fueron diagnosticadas con diabetes gestacional • historia de enfermedad cardiovascular • hipertensión (≥ 140/90 mmHg) o con tratamiento para la hipertensión • niveles de colesterol HDL <35 mg / dL (0,90 mmol / L) y / o niveles de triglicéridos> 250 mg / dL (2,82 mmol / L) • mujeres con síndrome de ovario poliquístico • inactividad física • Otras afecciones clínicas asociadas con la resistencia a la insulina (por ejemplo, obesidad severa, acantosis nigricans).
2. Para todos los pacientes, las pruebas deben comenzar a los 45 años
3. Si los resultados son normales, la prueba debe repetirse cada 3 años, salvo que sean diagnosticados de prediabetes o se encuentraen en un estado de riesgo (en ese caso las pruebas deben repetirse antes)

Tabla modificada de American Diabetes Association [29].

1.4 Epidemiología de la diabetes mellitus tipo 2

La diabetes mellitus constituye un importante problema de salud pública, una de las cuatro enfermedades no transmisibles (NCDs, non-communicable diseases) prioritarias de atención a nivel mundial. Tanto el número de casos como la prevalencia de diabetes han aumentado constantemente en las últimas décadas. En todo el mundo, se estima que 422 millones de adultos vivían con diabetes en 2014, en comparación con 108 millones en 1980[30].

La prevalencia global (estandarizada por edad) de la diabetes casi se ha duplicado desde 1980, pasando del 4,7% al 8,5% en la población adulta. Esto refleja un aumento en los factores de riesgo asociados, como el sobrepeso u obesidad. En la última década, la prevalencia de la diabetes ha aumentado más rápidamente en los países con ingresos bajos y medios que en los países con altos ingresos, siendo la mayoría de los casos

DMT2. La diabetes causó 1,5 millones de muertes en 2012 y los niveles de glucosa superiores a los óptimos causaron 2,2 millones de muertes adicionales al aumentar el riesgo de enfermedad cardiovascular y otras enfermedades. El 43% de estas 3.7 millones de muertes ocurren antes de los 70 años, siendo mayor en los países con bajos ingresos[31].

La **tabla 4.1** enumera las 15 principales causas de muerte para hombres, mujeres y ambos sexos combinados según las predicciones para el año 2030 a nivel mundial. Se estima que la diabetes subirá cuatro puestos en el ranking [32]. La **tabla 4.2** muestra las diez principales causas de muerte según los ingresos, siendo la diabetes la cuarta causa de muerte en los países de altos ingresos. La diabetes supone una gran carga económica al sistema sanitario mundial y a la economía mundial en general [33].

En 2013 la Asamblea Mundial de la Salud elaboró un proyecto con nueve objetivos voluntarios a alcanzar en el año 2025. Este fue acompañado por el plan de acción mundial 2013 - 2020 de la OMS para la prevención y control de las NCDs [34]. En ese marco de monitoreo mundial, la diabetes y sus principales factores de riesgo se reflejan fuertemente en el plan de acción global. Estos compromisos se profundizaron en 2015 con la adopción por la Asamblea General de las Naciones Unidas de la Agenda 2030 para el Desarrollo Sostenible [35]. En ese contexto, los distintos países acordaron tomar medidas para alcanzar objetivos ambiciosos para el año 2030, tales como, reducir un tercio la mortalidad prematura por NCDs, lograr una cobertura de salud universal y proporcionar acceso a los medicamentos esenciales.

Tabla 4.1. Cambios en los rankings para las 15 principales causas de muerte entre los años 2002 y 2030

Category	Disease or Injury	2002 Rank	2030 Ranks	Change in Rank
Within top 15	Ischaemic heart disease	1	1	0
	Cerebrovascular disease	2	2	0
	Lower respiratory infections	3	5	−2
	HIV/AIDS	4	3	+1
	COPD	5	4	+1
	Perinatal conditions	6	9	−3
	Diarrhoeal diseases	7	16	−9
	Tuberculosis	8	23	−15
	Trachea, bronchus, lung cancers	9	6	+3
	Road traffic accidents	10	8	+2
	Diabetes mellitus	11	7	+4
	Malaria	12	22	−10
	Hypertensive heart disease	13	11	+2
	Self-inflicted injuries	14	12	+2
	Stomach cancer	15	10	+5
Outside top 15	Nephritis and nephrosis	17	13	+4
	Colon and rectum cancers	18	15	+3
	Liver cancers	19	14	+5

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Tabla modificada de Mathers et al [32]

En España, el estudio Di@bet.es, utilizando una muestra representativa de toda la población española, mostró que la prevalencia de diabetes mellitus es del 13.8%, con un 6.8% sin diagnosticar [36].

1.5. Factores de riesgo (IMC, dislipidemia, hipertensión, tabaquismo, inactividad física, patrón dietético, genética)

El riesgo de DMT2 está determinado por la interacción de factores genéticos y metabólicos. El origen étnico, los antecedentes familiares de diabetes y la diabetes gestacional previa se combinan con la edad avanzada, el sobrepeso y la obesidad, la dieta no saludable, la inactividad física y el tabaquismo aumentando el riesgo de padecer la enfermedad [34]. La predisposición genética juega un papel importante en el riesgo de desarrollar DMT2.

Tabla 4.2. Diez principales causas de muerte, por grupo de ingresos para el año 2030

Income Group	Rank	Disease or Injury	Percent of Total Deaths
World	1	Ischaemic heart disease	13.4
	2	Cerebrovascular disease	10.6
	3	HIV/AIDS	8.9
	4	COPD	7.8
	5	Lower respiratory infections	3.5
	6	Trachea, bronchus, lung cancers	3.1
	7	Diabetes mellitus	3.0
	8	Road traffic accidents	2.9
	9	Perinatal conditions	2.2
	10	Stomach cancer	1.9
High-income countries	1	Ischaemic heart disease	15.8
	2	Cerebrovascular disease	9.0
	3	Trachea, bronchus, lung cancers	5.1
	4	Diabetes mellitus	4.8
	5	COPD	4.1
	6	Lower respiratory infections	3.6
	7	Alzheimer and other dementias	3.6
	8	Colon and rectum cancers	3.3
	9	Stomach cancer	1.9
	10	Prostate cancer	1.8
Middle-income countries	1	Cerebrovascular disease	14.4
	2	Ischaemic heart disease	12.7
	3	COPD	12.0
	4	HIV/AIDS	6.2
	5	Trachea, bronchus, lung cancers	4.3
	6	Diabetes mellitus	3.7
	7	Stomach cancer	3.4
	8	Hypertensive heart disease	2.7
	9	Road traffic accidents	2.5
	10	Liver cancer	2.2
Low-income countries	1	Ischaemic heart disease	13.4
	2	HIV/AIDS	13.2
	3	Cerebrovascular disease	8.2
	4	COPD	5.5
	5	Lower respiratory infections	5.1
	6	Perinatal conditions	3.9
	7	Road traffic accidents	3.7
	8	Diarrhoeal diseases	2.3
	9	Diabetes mellitus	2.1
	10	Malaria	1.8

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Tabla modificada de Mathers et al [32]

Alrededor del 84% de los adolescentes del Reino Unido con DMT2 tienen antecedentes familiares y un 56-71% un progenitor o hermano afectado [37, 38]. Estudios previos mostraron que los japoneses, los hispanos y los nativos americanos tienen los mayores riesgos de desarrollar DMT2 en la infancia [39-41]. Por otro lado, la incidencia de DMT2 aumenta en mujeres con diabetes mellitus gestacional previa o con síndrome de ovario poliquístico. Además, se estima que el sobrepeso y la obesidad, junto con la inactividad física, causan una gran proporción de la carga mundial de diabetes [42]. La gran mayoría de personas con DMT2 de inicio precoz son obesos (80-92%) en comparación con solo el 56% de los adultos mayores [38, 43]. El perímetro de cintura y el índice de masa corporal (IMC) se asocian con un mayor riesgo de DMT2, aunque la relación puede variar en diferentes poblaciones [44]. Las poblaciones del sudeste asiático, por ejemplo, desarrollan diabetes a un IMC más bajo que las poblaciones de origen europeo [45]. De hecho, el elevado consumo de grasas saturadas, bebidas azucaradas y un consumo inadecuado de fibra aumenta, especialmente en niños, el riesgo de tener sobrepeso y obesidad [46, 47], así como el riesgo de DMT2 [48-50]. El tabaco también aumenta el riesgo de desarrollar DMT2, incluso durante 10 años después de haber dejado de fumar [51]. Finalmente, el bajo nivel educativo [36] y socioeconómico está asociado con un peor estado de salud, mayores tasas de mortalidad y enfermedades cardiovasculares, así como el aumento de la prevalencia de diabetes [52].

2. FLEXIBILIDAD METABÓLICA.

2.1 Regulación del metabolismo de la glucosa

La concentración de glucosa en plasma depende de la cantidad de glucosa que entra a la circulación y de la que es metabolizada. La glucosa circulante proviene de tres fuentes: absorción intestinal (proviene de los alimentos), glucogenólisis (o degradación del glucógeno) y gluconeogénesis (formación de glucosa principalmente a partir de lactato y aminoácidos durante el estado de ayuno) (**Figura 1**). La glucogenólisis y la gluconeogénesis están reguladas por los niveles de glucagón, una hormona producida en las células α del páncreas.

Durante las primeras 8 a 12 horas de ayuno, el glucagón facilita la glucogenólisis, el mecanismo primario por el cual se obtiene la glucosa. Aunque la mayoría de los tejidos tienen la capacidad de hidrolizar el glucógeno, solo el hígado y los riñones contienen glucosa-6-fosfatasa, la enzima necesaria para la liberación de glucosa a la circulación. Durante períodos más largos de ayuno, se activa la gluconeogénesis para sintetizar glucosa a partir de precursores no glucídicos, aminoácidos, lactato, piruvato, glicerol y cualquiera de los intermediarios del ciclo de los ácidos tricarboxílicos (o ciclo de Krebs). Este proceso tiene lugar casi exclusivamente en el hígado, sólo un 10% en los riñones. Las hormonas que participan en la regulación de los niveles de glucosa circulantes son insulina, glucagón, amilina, GLP-1, polipéptido inhibidor gástrico (GIP), epinefrina, cortisol y hormona del crecimiento. La insulina y la amilina se derivan de las células beta pancreáticas, el glucagón de las células α, y el GLP-1 y GIP de las células L del intestino (**Tabla 5**) [53].

Después de comer, la insulina permite la entrada de la glucosa circulante al interior de las células y su almacenamiento en forma de lípidos y glucógeno. Además, activa la glucólisis, mientras que inhibe la gluconeogénesis y la liberación de glucagón [54, 55] (**Figura 1**).

Tabla 5. Efectos de las principales hormonas que particitan en la regulación de los niveles de glucosa circulantes

PÁNCREAS
Células-α: Glucagón
• Estimula la descomposición del glucógeno hepático almacenado
• Promueve la gluconeogénesis hepática
• Promueve la cetogénesis hepática
Células-beta: Insulina
• Regula el metabolismo de la glucosa y el almacenamiento de nutrientes ingeridos
• Promueve la absorción de glucosa por las células
• Suprime la secreción de glucagón posprandial
• Promueve la síntesis de proteínas y grasas
• Promueve el uso de glucosa como fuente de energía
Células-beta: Amilina
• Suprime la secreción de glucagón posprandial
• Reduce el vaciado gástrico
• Reduce la ingesta de alimentos y el peso corporal

INTESTINO ***Células-L : GLP-1***
• Mejora la secreción de insulina estimulada por glucosa (GSIS)
• Suprime la secreción de glucagón posprandial
• Reduce el vaciado gástrico
• Reduce la ingesta de alimentos y el peso corporal
• Promueve la salud de las células beta

Tabla modificada desde Aronoff et al [56]

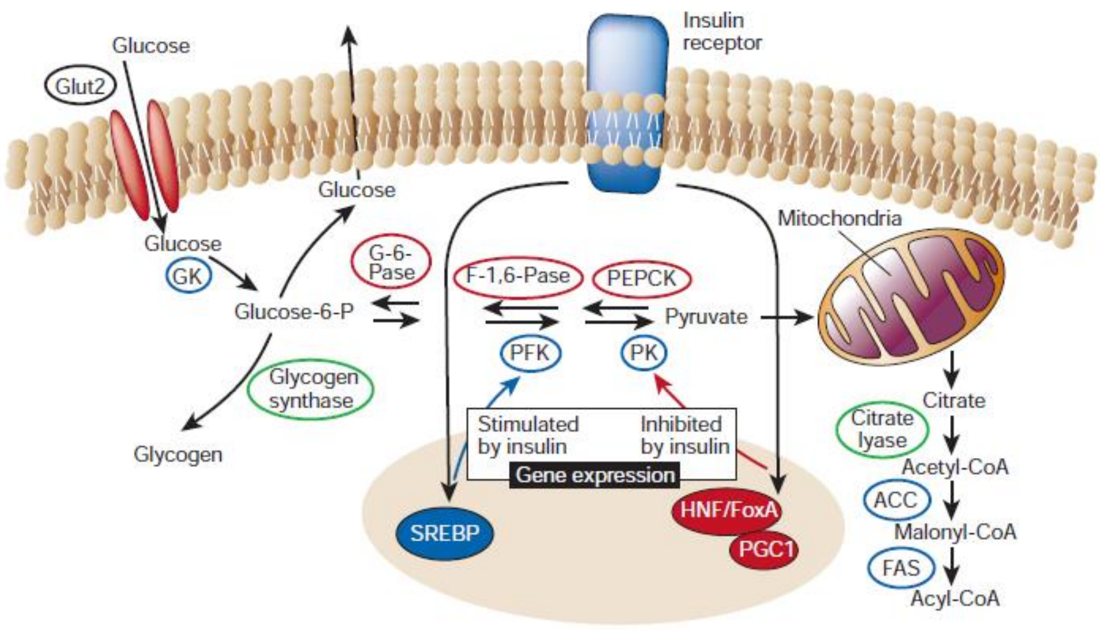

Figura 1: Regulación del metabolismo de la glucosa en el hígado. Modificado de Saltiel et al [57]

En el hepatocito, la insulina estimula la utilización y el almacenamiento de glucosa en forma de lípidos y glucógeno, a la vez que reprime la síntesis y liberación de la glucosa a la circulación sanguínea. La insulina estimula la expresión de genes que codifican las enzimas que participan en la glucólisis y en la síntesis de ácidos grasos (en azul), al tiempo que inhibe la expresión de las enzimas gluconeogénicas (en rojo). Estos efectos están mediados por una serie de factores de transcripción y cofactores, entre los que se encuentran la proteína de unión al elemento regulador de esteroles (SREBP) -1, el factor nuclear hepático (HNF) -4, proteínas de la familia FOx y el PPARgC1 (PGC1). Además, la insulina también regula las actividades de algunas enzimas, como la glucógeno sintasa y citrato liasa (en verde), a través de cambios en el estado de fosforilación. GK, glucoquinasa; Glucosa-6-P, glucosa-6-fosfato; G-6-Pase, glucosa-6-fosfatasa; F-1,6-Pase, fructosa-1,6-bisfosfatasa; PEPCK, fosfoenolpiruvato carboxiquinasa; PFK, fosfofructoquinasa; PK, piruvato quinasa; ACC, acetil-CoA carboxilasa; FAS, sintasa de ácidos grasos.

Cuando hay una alteración crónica del metabolismo de la glucosa y/o metabolismo de ácidos grasos, como ocurre en la obesidad, el exceso de estas moléculas genera toxicidad y alto riesgo de desarrollar enfermedades como diabetes mellitus tipo 2 y enfermedad cardiovascular.

2.2 Glucolipotoxicidad en el desarrollo de resistencia a insulina

La resistencia a la insulina constituye un trastorno metabólico muy complejo que tiene lugar como consecuencia de la acumulación de metabolitos lipídicos ectópicos, la activación de la respuesta a proteínas mal plegadas (UPR), el estrés oxidativo y la inflamación.

2.2.1 Glucotoxicidad

El término glucotoxicidad se refiere a los efectos perjudiciales lentos e irreversibles de los niveles de glucosa crónicamente elevados sobre la función de las células beta, lo que conlleva a una disminución de la síntesis de insulina.

En etapas tempranas del desarrollo de la DMT2, la función de las células beta comienza a ser anormal, lo que se evidencia por la pérdida de la respuesta de la insulina a la ingesta de alimentos [58]. La aparición de resistencia a la insulina a nivel periférico, junto con el fallo progresivo de las células beta y la disminución de la disponibilidad de insulina, amilina y GLP1 [59] desencadena el cuadro clínico de hiperglucemia característico de la diabetes [60]. Los principales mecanimos implicados en la alteración de la homeostasis de glucosa son:

Estrés del retículo endoplasmático (ER)

El aumento sostenido de la demanda de insulina debido a la hiperglucemia crónica desencadena estrés en el retículo endoplásmico (RE), lo que origina una acumulación de proteínas mal plegadas. Como consecuencia, se inicia la llamada respuesta a proteínas mal pleagadas (UPR), que tiene como objetivo restaurar la homeostasis en el RE. Cuando el UPR falla y no es capaz de disminuir el estrés del RE se activa la apoptosis celular [61]. Tres rutas fundamentales trabajan para reducir las proteínas mal plegadas en la luz del RE, aumentar la biogénesis de la membrana (ruta IRE1), detener la traducción de proteínas (ruta PERK) y mejorar la expresión de chaperonas (ruta ATF6)

[62]. En estados de resistencia a la insulina, las células beta intentan compensar aumentando la secreción de insulina, lo que podría saturar la capacidad del RE para su correcto procesamiento [63].

Estrés oxidativo y disfunción mitocondrial

Los niveles elevados crónicos de glucosa aumentan la generación de especies reactivas de oxígeno (ROS) en las mitocondrias de las células de los islotes pancreáticos, lo que induce estrés oxidativo y una peor respuesta a la secreción de insulina estimulada por glucosa (GSIS), viéndose afectada también la regulación de la masa celular [64]. Dado que la célula beta tiene niveles muy bajos de enzimas antioxidantes, es particularmente vulnerable al estrés oxidativo [60].

El aumento de los niveles de glucosa y ROS activa la proteína de desacoplamiento 2 (UCP2), que reduce el potencial de la membrana mitocondrial dando lugar a la producción de calor. Aunque el desacoplamiento de la fosforilación oxidativa puede proteger a las células beta contra la producción adicional de ROS y el estrés oxidativo, también se reduce la síntesis de ATP, necesaria para la secreción de insulina. Por lo tanto, el aumento de la expresión de UCP2 puede contribuir a los efectos nocivos de ROS sobre la función de las células beta [65].

Inflamación y apoptosis celular

Estudios previos demostraron que la producción de citoquinas proinflamatorias está mediada por ROS. La hiperglucemia crónica desencadena estrés oxidativo e inflamación, lo que puede inducir cambios en la regulación de la expresión génica dando lugar a alteraciones en la secreción de insulina y un aumento de la apoptosis de las células beta [66]. Existen múltiples vías que median la respuesta inflamatoria en el desarrollo de resistencia a la insulina, pero a menudo, convergen en la activación de JNK1. La activación de JNK1 también juega un papel destacado en el mecanismo por el cual el UPR (a través de la activación de IRE1α) desencadena resistencia a la insulina [67], lo que puede estar relacionado con la alteración del metabolismo lipídico. Específicamente, la activación de JNK1 en adipocitos aumenta la liberación de citoquinas que podría estar asociada con un aumento de la lipólisis [67] .

2.2.2. Lipotoxicidad

El término de lipotoxicidad hace referencia a los efectos nocivos de las concentraciones elevadas de ácidos grasos plasmáticos (AGL) sobre la función de las células beta. Estudios previos han mostrado que el aumento en los ácidos grasos libres altera la GSIS, la expresión génica de la insulina y aumenta la apoptosis y necrosis de células beta in vitro, tanto en líneas celulares como en islotes pancreáticos aislados (humanos) [68, 69].

Se han propuesto varios mecanismos sobre cómo los AGL afectan a la función de las células beta. En primer lugar, inducen estrés en el RE, muy probablemente como consecuencia de la sobreestimulación de la esterificación de los NEFA (del inglés "non-esterified fatty acids"), lo que reduce la capacidad del RE para otros procesos [70]. En segundo lugar, se ha demostrado que los NEFA inducen estrés oxidativo tanto in vitro como in vivo [71, 72]. Por último, los AGL, y especialmente los saturados como el palmitato, pueden servir como sustrato para la formación de ceramidas y otros metabolitos, los cuales inducen apoptosis y reducen la expresión del gen de la insulina [71].

Cambios en la absorción de ácidos grasos, la lipogénesis y el gasto de energía pueden afectar a la deposición de lípidos ectópicos. Los primeros estudios realizados por Randle y colaboradores [73] en corazón y diafragma de roedores sugirieron que los ácidos grasos alteran el metabolismo de la glucosa en el músculo mediado por la acción de la insulina a través de la inhibición de la piruvato deshidrogenasa, lo que conduce a una reducción de la oxidación de glucosa. En ratas normales, infusiones de heparina y lípidos, resultó en resistencia muscular a la insulina, asociada a un aumento en la acumulación de diacilglicerol (DAG), lo que conlleva a una activación de la proteína quinasa C (PKC), un deterioro en la señalización de la insulina y en la captación de glucosa muscular sin mostrar cambios en los niveles de triglicéridos [74]. En humanos la relación entre la activación de PKC mediada por DAG y la resistencia a la insulina también ha sido demostrada [75, 76].

La acumulación de lípidos ectópicos también ocurre en el hígado. Varios estudios en humanos demostraron la fuerte asociación entre la enfermedad hepática no alcohólica (NAFLD) per se y la resistencia a la insulina hepática [77]. Estudios previos mostraron que la esteatosis hepática y la resistencia a la insulina se asociaron con alteraciones en la expresión de proteínas transportadoras de lípidos [78, 79], y a una sobreexpresión de la lipoprotein lipasa (LpL) [78]. Al igual que en el músculo, la acumulación de DAG en el hígado activa la expresión de las PKC e induce una alteración de la señalización de la insulina [80, 81]. La acumulación de ceramidas se ha asociado con la alteración de la ruta Akt [67]. En individuos prediabéticos se ha visto un aumento significativo de las dihydroceramidas, por lo que han sido propuestas como biomarcadores tempranos de enfermedad [82].

Por lo tanto, la acumulación de diacilgliceroles y ceramidas en el hígado y el músculo parece ser una vía común que conduce a la alteración de la señalización de la insulina y a resistencia a la insulina. Además, la acumulación de grasa ectópica a nivel del páncreas [83] puede contribuir a la disfunción de las células beta [84]. En la **figura 2** se representa la relación entre la acumulación de acidos grasos y la funcionalidad de las células beta.

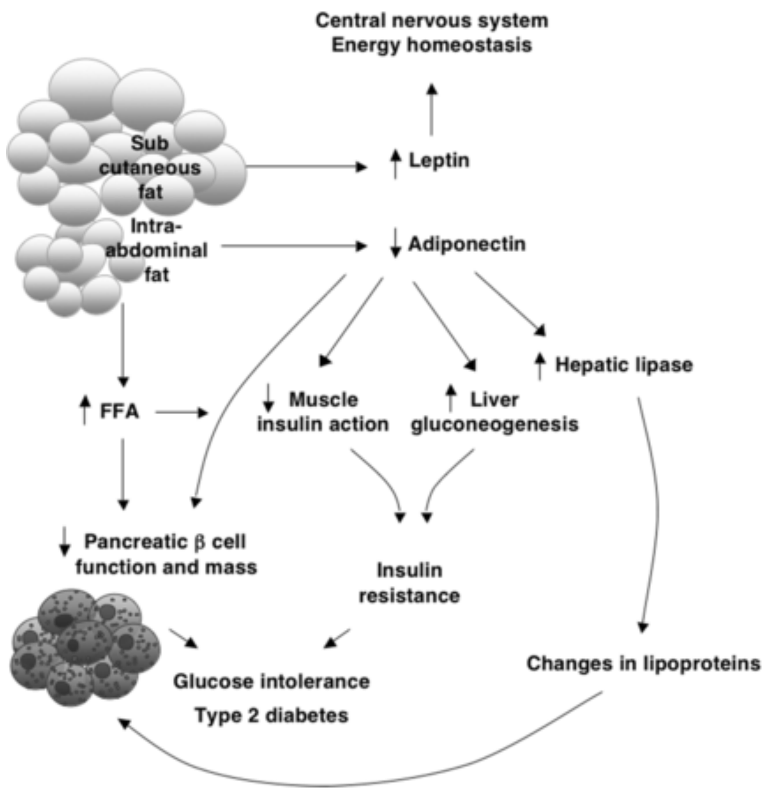

Figura 2: Efecto de los niveles elevados de ácidos grasos sobre la función y masa de las células beta pancreáticas y la sensibilidad a la insulina en la patogénesis de la diabetes tipo 2. Modificado de Cnop et al [71]

El aumento en el depósito de grasa intraabdominal disminuye los niveles de adiponectina y aumenta los de NEFA, lo que inhibe los efectos de la insulina en el hígado y en el músculo y desencadena un aumento de la gluconeogénesis y una captación de glucosa menos eficiente. Así, los niveles bajos de adiponectina inducen resistencia hepática a la insulina e incrementan la actividad de la lipasa hepática, reduciendo el colesterol HDL y aumentando las partículas de LDL pequeñas y densas [85], tal y como se observa en el síndrome metabólico.

En resumen, los niveles crónicos elevados de glucosa y las acumulaciones de grasa inducen resistencia a la insulina que favorecen el desarrollo de intolerancia a la glucosa.

2.3. Fisiología de las células beta pancreáticas

El páncreas es una glándula endocrina que se compone de agregaciones de cuatro tipos principales de células, células alfa (secretan glucagón), células beta (secretan insulina), células delta (secretan somatostatina) y células F (secretan polipétido pancreático). Los agregados celulares se parecen a "islas" de células y se denominan "islotes de Langerhans" [86]. El islote es generalmente de forma oval y la distribución de las células endocrinas dentro de un islote es similar en la mayoría de los mamíferos, y se caracteriza por un núcleo de células β rodeadas por un manto de células no-β. La vía (s) y el mecanismo (s) que subyacen a la proliferación de las células beta de los islotes en los seres humanos son en gran parte desconocidos. Por ejemplo, en roedores, varios factores se han visto implicados en el crecimiento de las células beta, incluidos los nutrientes, como la glucosa, hormonas como el péptido similar a glucagón (GLP-1) y varios factores de crecimiento, incluidos los factores de crecimiento similares a la insulina (IGF), el factor de crecimiento de fibroblastos y el factor de crecimiento de hepatocitos / factor de dispersión [87]. La función principal de las células beta es segregar cantidades apropiadas de insulina bioactiva, en respuesta a los nutrientes, hormonas y estímulos nerviosos, para mantener los niveles de glucosa en plasma en un rango fisiológico ajustado para el funcionamiento óptimo de todos los tejidos del cuerpo. Teniendo en cuenta la arquitectura anatómica del islote, es probable que la célula beta también module la función de las células vecinas a través de interacciones paracrinas / autocrinas [88].

2.3.1 Biosíntesis de insulina

La insulina, que se produce en las células beta, es un regulador crítico del metabolismo. Se sintetiza como preproinsulina y es procesada a proinsulina. La proinsulina luego se convierte en insulina y péptido C y se almacena en gránulos de secreción junto con la amilina a la espera de su liberación bajo demanda. La síntesis de insulina está regulada tanto a nivel transcripcional como traduccional [89]. Proteínas tales como PAX6, PDX-

1, MafA y NeuroD1 regulan la transcripción de insulina, mientras que la estabilidad del ARNm de preproinsulina y sus regiones no traducidas controlan la traducción. La secreción de insulina implica una secuencia de eventos en las células beta que conducen a la fusión de los gránulos secretores con la membrana plasmática, principalmente en respuesta a glucosa, aunque también otros nutrientes como los ácidos grasos libres y los aminoácidos pueden aumentar la secreción de insulina inducida por la glucosa. Además, varias hormonas, como la melatonina, los estrógenos, la leptina, la hormona de crecimiento y el GLP1 también regulan la secreción de insulina. Por lo tanto, la célula beta es un centro metabólico que conecta el metabolismo de los nutrientes y el sistema endocrino. Aunque un aumento en [Ca2 + intracelular] es la señal primaria, los mecanismos dependientes de la señalización de AMP cíclico también son críticos en la regulación de la secreción de insulina [89].

2.3.2 Regulación de la secreción de insulina

En sujetos sanos, la liberación de insulina tiene lugar para satisfacer la demanda metabólica, así, las células beta detectan cambios en la concentración de glucosa en plasma y responden liberando la cantidad correspondiente de insulina [90]. Las células beta se agrupan en islotes, una red densa con pequeños vasos sanguíneos que reciben 10 veces más sangre que las células de las regiones exocrinas circundantes. Los capilares que rodean los islotes presentan pequeños poros llamados "*fenestrae* " que permiten un mayor intercambio de nutrientes entre la circulación y los tejidos circundantes. Esta estructura mejora la permeabilidad, lo que permite el acceso ilimitado a los nutrientes para que las células beta puedan detectar el estado nutricional rápidamente. Las fenestraciones también permiten la rápida difusión de insulina a la sangre [91]. Además de la glucosa, algunos aminoácidos y ácidos grasos también regulan la secreción de insulina. En la **figura 3** se muestra una ilustración esquemática de la secreción de insulina regulada por nutrientes.

Parece ser que las células beta no contienen receptores de glucosa en su membrana plasmática, pero están equipadas con varios sensores que detectan lo niveles de glucosa circulante, como el transportador de glucosa 2 (GLUT2). A diferencia del GLUT4, que se expresa principalmente en células musculares y adiposas, la movilización de GLUT2 a la membrana plasmática es independiente de la insulina y muestra una baja afinidad por el sustrato, asegurando una alta afluencia de glucosa [89].

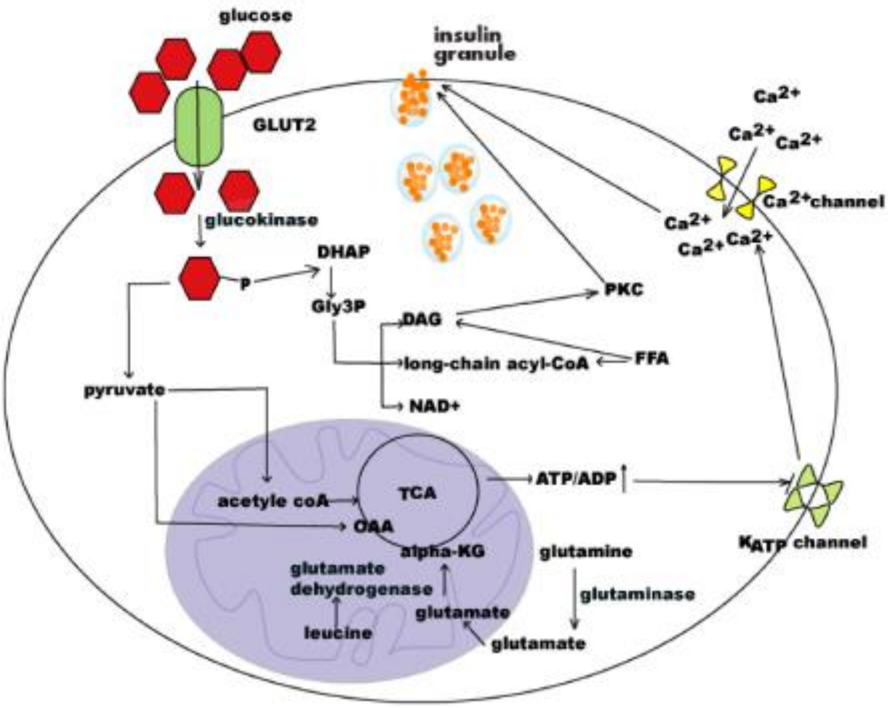

Figura. 3 Secreción de insulina regulada por nutrientes. Modificado de Fu et al [92]

Una vez que la glucosa entra al interior de la céula beta, es fosforilada por la glucoquinasa, un subtipo de hexoquinasa, a glucosa-6-fosfato (G6P). La glucoquinasa tiene menor afinidad por la glucosa que otras hexoquinasas y no es inhibida por producto, por lo que es el paso limitante en el metabolismo de la glucosa en las células beta y se considera, también, un importante sensor de glucosa [91].

El producto final de la glucólisis es el piruvato, que posteriormente es oxidado para obtener ATP en el ciclo de Krebs o ciclo de los ácidos tricarboxílicos (TCA), la principal vía asociada a la liberación de insulina, en la que participan los canales de potasio dependientes de ATP. El aumento intracelular del ratio ATP/ADP abre los canales de potasio lo que produce una despolarización de la membrana plasmática, una apertura de los canales de Ca2 + dependientes del voltaje, la afluencia de Ca2 + y la activación de la exocitosis de los gránulos que contienen la insulina. Algunos productos derivados del ciclo TCA, como NADPH, malonil-CoA y glutamato, pueden actuar como señales para la secreción de insulina. Se ha visto que estas moléculas aumentan la secreción de insulina mediada por los canales de potasio dependientes de ATP [93, 94].

Además del ciclo TCA, la G6P también puede ser metabolizada hacia fosfato de dihidroxiacetona (DHAP) y finalmente, glycerol-3 fosfato (Gly3P). El Gly3P puede metabolizarse en la vía de degradación de triglicéridos hasta acyl-CoA y DAG que también pueden aumentar la secreción de insulina.

2.3.3 Ácidos grasos y secreción de insulina

Los ácidos grasos libres (AGL) mejoran la GSIS para compensar la resistencia a la insulina presente en la diabetes tipo 2 [95, 96]. Se ha visto que las células beta tienen un receptor de ácidos grasos libres (FFAR) [97, 98] y pierden la capacidad de secretar insulina cuando no hay ácidos grasos disponibles, lo que se puede revertir administrándolos de forma exógena [99, 100].

La acil-CoA, producto del metabolismo lipídico, contribuye a acilar proteínas esenciales en la fusión de los gránulos de insulina con la membrana celular [101] y los DAG, otro producto de la lipólisis, activan la proteína quinasa C y Mun-13, para promover la secreción de insulina [102] [103].

2.3.4 Aminoácidos y secreción de insulina

Los aminoácidos de forma individualizada y a concentraciones fisiológicas no estimulan la secreción de insulina, sin embargo, ciertas combinaciones de aminoácidos a concentraciones fisiológicas o superiores pueden aumentar la GSIS [104]. Por ejemplo, la glutamina por si sola no estimula la secreción de insulina, pero en combianción con leucina puede aumentar la GSIS [105].

Ciertos aminoácidos como alanina y glutamina, se liberan a la sangre y sirven como potentes secretagogos del glucagón. Los aminoácidos de la dieta también pueden inducir la secreción de insulina a través de mecanismos dependientes de incretinas. El GIP y el GLP1 son las dos principales hormonas incretinas secretadas por el tracto gastrointestinal. La ingestión de nutrientes en el intestino, incluida la glucosa y los aminoácidos, estimula la secreción de estas hormonas desde las células K intestinales y las células L. Las incretinas actúan directamente sobre las células beta uniéndose a sus receptores específicos de la superficie celular, aumentando la GSIS [106, 107],

2.4. Disfunción de las células beta

La disfunción de la célula beta se trata de un proceso secuencial que comienza con la disminución progresiva de la secreción de insulina, seguido por una disminución en la masa celular y finalmente la apoptosis celular [108, 109]. Procesos tales como la inflamación, el estres oxidativo y de reticulo endoplásmico, así como la acumulación de acidos grasos, no están unicamente relacionados con la resistencia a la insulina, sino también con la disfución de la células beta y por tanto, con en el desarrollo de la DMT2 [110].

2.4.1 Factores implicados en la disfunción de la célula beta

Como se ha mencionado en apartados anteriores, tanto la obesidad como la resistencia a la insulina se caracterizan por un estado inflamatorio. La secreción de citoquinas proinflamatorias en un proceso mediado por la síntesis de ROS y por el estrés mitocondrial conllevan a muerte de las células beta [111, 112]. Asi mismo, la hiperglucemia crónica supera la capacidad glicolítica de las células beta lo que genera estrés oxidativo e inflamación, alterándose la secreción de insulina y dando lugar a un aumento en la apoptosis cellular [66]. El estrés oxidativo provoca daños en los diferentes orgánulos celulares, particularmente las proteínas mitocondriales, los lípidos y los ácidos nucleicos [113], contribuyendo además al estrés del retículo endoplásmico (ER) [114, 115] y la autofagia [116]. Las células beta son altamente dependientes de la producción de ATP para secretar insulina y son muy vulnerables al exceso de ROS porque tienen baja expresión de enzimas antioxidantes [113]. El imbalance o la reducida disponibilidad de nutrientes, los pequeños y repetidos incrementos en la producción de ROS, la baja síntesis de ATP y el inadecuado balance de antioxidantes conduce a la disfunción de la célula beta [117].

Por otra parte, la hiperglucemia persistente junto con el aumento en ácidos grasos saturados induce un estado glucolipotóxico que es perjudicial para las células beta al aumentar el estrés oxidativo, reduciendo la síntesis y la secreción de insulina, lo que compromete la estructura y la función celular [118].

Los efectos de los ácidos grasos sobre la funcionalidad de la célula beta depende del tiempo de exposición. La exposición a corto plazo aumenta la GSIS, lo que produce una mayor secreción de insulina después de una comida mixta y permite el almacenamiento de exceso de calorías en forma de grasa [119]. Por el contrario, la exposición a ácidos grasos a largo plazo suprime la GSIS, altera el metabolismo de la glucosa, reduce la biosíntesis de insulina y conduce a una pérdida de masa de células beta [60].

La inflamación de los islotes en la diabetes tipo 2 se atribuye a la sobrecarga de nutrientes que conduce al agotamiento metabólico de las células beta [120]. Estudios previous han mostrado como el palmitato, un ácido graso saturado, induce disfunción de las células beta in vivo mediante la activación de procesos inflamatorios dentro de los islotes [121]. Además, se ha demostrado que el tratamiento con palmitato incrementa la expresión de las principales citoquinas implicadas en la disfunción de las células beta [122], IL-6, IL-8 (CXCL1), IP10, MCP1 (CCL2) y MIP1A (CCL3) [123].

2.5. Pérdida de flexibilidad metabólica como desencadenante de enfermedad

La fisiología normal de nuestro organismo mantiene una maquinaria bien orquestada que nos permite adaptarnos a un entorno en constante cambio, en el que la alimentación tiene un papel fundamental. A esa capacidad de respuesta del organismo se le conoce como "flexibilidad fenotípica", que es clave para mantener la homeostasis general y, por lo tanto, la salud. Los procesos y mecanismos que forman la base de la flexibilidad fenotípica son la biosíntesis de ATP, el estrés oxidativo, respuesta inflamatoria, función inmune, reparación del ADN y la apoptosis, entre otros [124]. Así, la capacidad de adaptarse en tiempo y lugar a las alteraciones en los factores externos, como las condiciones ambientales, se denomina flexibilidad fenotípica. Las condiciones de estrés crónico pueden inducir procesos de adaptación que van más allá de los límites de la flexibilidad fenotípica normal, lo que conduce a la inflexibilidad progresiva y al inicio de la enfermedad [124].

Varios procesos y mecanismos están involucrados en la flexibilidad fenotípica como la regulación metabólica de triglicéridos, la glucosa, un equilibrio inflamatorio óptimo, la regulación del estrés oxidativo y flexibilidad metabólica muscular entre otros [125] Curiosamente, la mayoría de estos mecanismos se ven alterados en pacientes obesos, por lo que requieren un alto grado de flexibilidad para compensar y mantener la homeostasis.

Un ejemplo clave de la pérdida de flexibilidad metabólica es el desarrollo de diabetes mellitus tipo 2. Generalmente, transcurren años desde un deterioro inicial de la acción de la insulina en los tejidos periféricos, con hiperglucemia e hiperinsulinemia hasta una pérdida progresiva de la funcionalidad de las células beta [124]. De forma fisiológica un aumento en los niveles de glucosa en plasma desencadena la puesta en marcha de un serie de mecanismos comensatorios. En primer lugar, se liberan al torrente sanguíneo las hormonas gastrointestinales CCK, GIP, GLP1 y PYY que inducen la secreción de insulina y pétido C [126] e inhiben la secreción de glucagón. La insulina controla la entrada de glucosa en el músculo y el tejido adiposo mediante el reclutamiento del transportador de glucosa GLUT4 en la membrana plasmática, mientras que en el hígado, se activa la producción de glucógeno [124]. Los niveles elevados de glucosa de forma crónica conducen al fallo en el mantenimiento de la homeostasis metabólica, una inflexibilidad progresiva y la aparición de enfermedad.

Además, la DMT2 se ha asociado con un metabolismo lipídico postprandial alterado, con un retraso significativo en el aclaramiento de lipoproteínas, TG y quilomicrones [127]. Los lípidos se ensamblan en quilomicrones con la ayuda de apolipoproteínas sintetizadas en las células epiteliales y se liberan a la linfa. Posteriormente, son almacenados en el tejido adiposo, un tejido con capacidad de expansión, proceso fundamental en la flexibilidad metabólica. El fracaso en la expansión del tejido adiposo en la obesidad da lugar a enfermedades como la diabetes tipo 2 y otras enfermedades metabólicas [128]. Una vez que se alcanza la capacidad máxima del individuo de almacenar grasa en los adipocitos, los lípidos comienzan a acumularse en otros tejidos, como el hígado y los músculos [128] lo que conduce a resistencia a la insulina. Un biomarcador de esta inflexibilidad metabólica es el grado de respuesta postprandial a los triglicéridos, lo que representa un estado altamente dinámico, con adaptaciones metabólicas continuas [129]. Los pacientes metabólicamente sanos muestran una respuesta posprandial más baja a los triglicéridos (TG) plasmáticos y las lipoproteínas ricas en TG, en comparación con aquellos metabólicamente enfermos, independientemente de si eran obesos o no. [125].

Por otra parte, la homeostasis de proteínas, degradación y síntesis, está sujeta a la regulación por numerosas hormonas. La insulia promueve la síntesis de proteínas y es la hormona que ejerce un mayor efecto en la regulación del balance proteico, en comparación con todas las demás hormonas, como la hormona de crecimiento (IGF), la epinefrina, la testosterona o las hormonas tiroideas. A diferencia de la insulina, el glucagón, los glucocorticoides y las catecolaminas activan la degradación de proteínas [124]. Una alta ingesta de proteínas, ya sea aguda o crónica, tiene efectos complejos en el sistema metabólico, ya que fuerza a utilizar los aminoácidos como sustratos energéticos, reduciendo así la necesidad de quemar lípidos e hidratos de carbono, lo que puede ocasionar glucotoxicidad, lipotoxicidad y resistencia a la insulina [124].

2.6. Evaluación de la sensibilidad y la resistencia a la insulina mediante pruebas dinámicas

La resistencia a la insulina desempeña un papel fundamental en la fisiopatología de la DMT2 y está estrechamente asociada con importantes problemas de salud pública, como obesidad, enfermedad cardiovascular, y un conjunto de anomalias que definen el síndrome metabólico [130-132]. Además, la resistencia a la insulina se asocia comúnmente con adiposidad visceral, intolerancia a la glucosa, hipertensión, dislipidemia, estado hipercoagulable, disfunción endotelial y marcadores elevados de inflamación. Por lo tanto, es de gran importancia desarrollar herramientas para cuantificar la sensibilidad / resistencia a la insulina en humanos, que puedan utlizarse para investigar eficientemente la epidemiología, los mecanismos fisiopatológicos, e interpretar los resultados de intervenciones terapéuticas [133].

La resistencia a la insulina se define como una disminución de la sensibilidad y / o capacidad de respuesta del organismo a la acción de la insulina para regular los niveles de glucosa en sangre, inhibir la producción hepática de glucosa y la lipólisis en el tejido adiposo. Para evaluar de forma rigurosa la sensibilidad a la insulina, es necesario comparar curvas de dosis respuesta. El método de "glucose clamp" es el estándar de referencia para la medida directa de la sensibilidad a la insulina. De entre el grupo de métodos más simples, QUICKI y Log (HOMA) son los mejores y más ampliamente validados. Por otro lado, las pruebas dinámicas son útiles si se necesita información tanto de la secreción de insulina como de la acción de la insulina (Tabla 6) [134].

Tabla 6: Métodos para evaluar la sensibilidad y la resistencia a la insulina en humanos

Method	Measure of Insulin sensitivity
Direct Measures	
Hyperinsulinemic Euglycemic Glucose Clamp	Average glucose infusion rate (GIR) = glucose disposal rate (M). $SI_{Clamp} = M/(G \times \Delta I)$, where M is normalized for G (steady-state blood glucose concentration) and ΔI (difference between fasting and steady-state plasma insulin concentrations)
Insulin-suppression Test (IST)	Steady-state plasma glucose (SSPG) concentrations during constant infusions of insulin and glucose with suppressed endogenous insulin secretion
Indirect Measures	
Minimal Model Analysis of Frequently Sampled Intravenous Glucose Tolerance Test (FSIVGTT)	Minimal model uniquely identifies model parameters that determine a best fit to glucose disappearance during the modified FSIVGTT. S_I : fractional glucose disappearance per insulin concentration unit; S_G (glucose effectiveness): ability of glucose *per se* to promote its own disposal and inhibit HGP in the absence of an incremental insulin effect (i.e., when insulin is at basal levels).
Simple Surrogate Indexes	
Surrogates Derived from Fasting Steady-state Conditions	
The Homeostasis Model Assessment (HOMA)	HOMA-IR = [(Fasting Insulin (μU/mL)) X (Fasting Glucose (mmol/L))]/22.5
Quantitative Insulin Sensitivity Check Index (QUICKI)	QUICKI = 1/[Log (Fasting Insulin, μU/ml) + Log (Fasting Glucose, mg/dl)]
Surrogates Derived from Dynamic Tests (OGTT)	
Matsuda Index	$ISI(Matsuda) = 10000/\sqrt{[(G_{fasting}\ (mg/dl) \times I_{fasting}\ (\mu U/ml) \times (G_{mean} \times I_{mean})]}$
Gutt Index - $ISI_{(0, 120)}$ $(mg.l^{2}.mmol^{-1}.mIU^{-1}.min^{-1})$	$ISI_{(0, 120)} = 75000 + (G_0\text{-}G_{120})(mg/l) \times 0.19 \times BW / 120 \times G_{mean\ (0,\ 120min)}\ (mmol/l) \times \text{Log}\ [I_{mean\ (0,\ 120min)}](mU/l)$

Tabla modificada de Ranganath et al [134]. ***Gmean**, concentración media de glucosa en plasma durante el OGTT; **Ir**, concentración de glucosa en plasma durante el ayuno; **G120**, concentración de glucosa en plasma a los 120 min; **Imean**, concentración media de insulina durante OGTT; **I0**, concentración de insulina en plasma durante el ayuno; **I120**, concentración de insulina en plasma a los 120 min.*

2.6.1 Medidas directas de la sensibilidad a la insulina

Clamp euglucémico hiperinsulinémico

Procedimiento: Desde su descripción inicial por DeFronzo y colaboradores esta técnica está considerada como el «estándar de oro» del estudio de la insulinorresistencia en humanos [135]. Por tanto, la validación de los métodos surgidos posteriormente para la evaluación de la resistencia insulínica debe pasar necesariamente por la comparación de sus resultados con los obtenidos por el clamp. El método consiste en la infusion endovenosa de insulina para mantener una insulinemia permanentemente elevada por encima de la correspondiente al período de ayuno; simultáneamente se van realizando determinaciones de glucemia cada 2-5 minutos para infundir glucosa a un ritmo tal que permita mantener una glucemia alrededor de 5 mmol/L de forma estable. El ritmo de infusión de glucosa necesario será proporcional a la sensibilidad a la insulina, y por tanto, inversamente proporcional a la insulinresistencia. El resultado se expresa como mg/kg/min o bien en forma de coeficiente, siendo el valor 1 el resultado del clamp promedio en el grupo de edad de menos de 35 años y con 90-110% del peso ideal.

Ventajas y limitaciones: Sus ventajas son múltiples. Mide la acción de la insulina sin que puedan interferir factores de confusión derivados de la secreción endógena de insulina o de niveles variables de glucemia. Su grado de reproducibilidad es el más alto de todas las técnicas disponibles, situándose su coeficiente de variación intraindividual entre el 5 y el 15% según los autores [136, 137]. Además permite, con un grado mayor de complejidad de la técnica, incorporar trazadores radiactivos como la glucosa tritiada para diferenciar entre los dos grandes componentes de la resistencia a la insulina: la captación muscular de glucosa y la generación de la misma por la neoglucogénesis hepática [138].

A pesar de ser la técnica más precisa y reproducible, no está exenta de *inconvenientes* [136]. La situación que se reproduce es fija en cuanto a la relación entre glucemia e insulinemia, y por tanto nada fisiológica, ya que en realidad son parámetros dependientes entre sí pero en constante cambio en el organismo. Además, su complejidad técnica hace que esté al alcance de pocos laboratorios y que no sea factible su uso en grandes muestras de población. Por último, y aunque parezca sorprendente, no está estandarizado su uso con lo que cada laboratorio utiliza su propia variante. Estos rasgos diferenciales suelen afectar a la dosis de insulina utilizada (la más habitual es 1mU/kg/min [136], pero existen otras, como por ejemplo 50 mU/min/70kg [139]), al grado de tecnificación para el cálculo e infusión de glucosa requerida (desde manual hasta utilizando complejos sistemas computarizados), y al período de tiempo escogido para calcular la media de infusion de glucosa necesaria (variando entre los últimos 40 y 100 minutos, aunque lo que parece más fiable son los últimos 60 minutos [136]).

Test de supresión de insulina (IST)

El IST proporciona una medida directa de la capacidad de la insulina exógena necesaria para metabolizar una carga de glucosa intravenosa suprimiendo la secreción endógena de insulina. Cuando se alcanza un estado estable, se determian las concentraciones de glucosa e insulina en plasma. La concentración de glucosa en plasma será mayor en sujetos resistentes a la insulina y menor en sujetos sensibles a la insulina [134].
Ventajas y limitaciones: Este método mide de forma directa y altamente reproducible las acciones metabólicas de la insulina, requiere menos mano de obra y es menos exigente desde el punto de vista técnico que el "glucose clamp". Sin embargo, no es práctico aplicar el IST en grandes estudios epidemiológicos o en el entorno de atención clínica. En condiciones ideales determina principalmente la sensibilidad a la insulina del músculo esquelético y no está diseñado para reflejar la sensibilidad a la insulina hepática.

2.6.2 Medidas indirectas de la sensibilidad a la insulina

Minimal model analysis of frequently sampled intravenous glucose tolerance test (FSIVGTT)

Fue desarrollado por Bergman [140] en un intento por encontrar una forma más simple que el clamp de estudio de la resistencia y secreción de insulina,. La base metodológica es un test de tolerancia endovenosa a la glucosa. Consiste en una infusión en bolo de una dosis suprafisiológica de glucosa (0,3 g/kg); a partir de ese momento se realizan extracciones seriadas durante 3 horas (hasta el minuto 30 cada 1-2 minutos, después cada 10-20 minutos, en total unas 30 extracciones) para determinación de glucemia e insulinemia. [138]. Los datos obtenidos son procesados por un programa informático, [141], que calcula el índice de sensibilidad a la insulina (SI) y el índice de secreción de insulina [141]. AIR ("acute insulin response").

Ventajas y limitaciones: En general, sus resultados ofrecen una buena correlación con los del clamp, llegando en algunos estudios a ser el coeficiente de correlación de 0,89 [142], y ha sido un método muy utilizado en diferentes poblaciones [143, 144].

Es más fácil de realizar porque requiere menos mano de obra, y no hay infusiones intravenosas que necesiten ajustes constantes y se obtienen excelentes predicciones del aclaramiento de glucosa. Sin embargo, también tiene inconvenientes. El más importante es que su precisión y validez decae mucho a medida que desciende la capacidad secretora de insulina del individuo, por lo que es poco útil en personas diabéticas [145, 146]. El motivo es que este tipo de pacientes tiene disminuida la secreción inicial de insulina, importante componente del modelo matemático. Aunque en un intento por salvar este obstáculo se han definido variantes añadiendo tolbutamida o insulina exógena durante el test, este aspecto sigue siendo el más serio inconveniente del método[147] . Por último, y aunque no tanto como el clamp, sigue siendo un procedimiento complejo que exige extracciones y procesamiento computarizado.

Test de tolerancia oral a la glucosa (OGTT)

El test de tolerancia oral a la glucosa (OGTT) es una prueba simple ampliamente utilizada en la práctica clínica para diagnosticar la intolerancia a la glucosa y la diabetes mellitus tipo 2 [148]. Después de una noche de ayuno, se toman muestras de sangre para determinar las concentraciones de glucosa e insulina a los 0, 30, 60 y 120 minutos después de una sobrecarga oral de glucosa estándar (75 g). La tolerancia oral a la glucosa refleja la eficiencia del cuerpo para eliminar la glucosa después de una sobrecarga glucosa o la ingesta de comida.

Ventajas y limitaciones: El OGTT imita la dinámica de glucosa e insulina de las condiciones fisiológicas de una forma más real que las condiciones utilizadas en el clamp y los métodos de IST o FSIVGTT. Sin embargo, es importante reconocer que la tolerancia a la glucosa y la sensibilidad a la insulina no son conceptos equivalentes. Además de las acciones metabólicas de la insulina, la secreción de insulina, los efectos de la incretinas y otros factores contribuyen de manera importante a la tolerancia a la glucosa. Por lo tanto, las pruebas de OGTT y de tolerancia a las comidas brindan información útil acerca de la tolerancia a la glucosa pero no la sensibilidad / resistencia a la insulina per se.

2.6.3 Índices para evaluar la sensibilidad / resistencia a la insulina

Derivados del estado de ayunas

Modelo homeostático con datos basales (HOMA: Homeostasis model assessment)

Fue desarrollado por el grupo de Turner en la primera mitad de los años 80 [149]. Su base metodológica es un modelo matemático desarrollado a partir de datos conocidos en humanos en cuanto a la relación de interdependencia entre la glicemia y la insulinemia (homeostasis). Este modelo se basa en que cuando existe un déficit secretor de insulina, la insulinemia puede mantenerse cerca de lo normal a expensas de tener una glicemia basal elevada y viceversa, cuando existe resistencia a la insulina, la glucemia basal tiende a mantenerse cerca de lo normal gracias a una hiperinsulinemia compensadora [150].

Ventajas y limitaciones: Como es obvio, su gran sencillez (una extracción basal) es su ventaja más importante. Además, para calcular el índice de resistencia se pueden utilizar fórmulas relativamente sencillas derivadas de la original más compleja (p.ej. insulinemia (mU/mL) x glicemia (mmol/L) / 22,5) [151]. Sus resultados guardan una buena correlación con los del clamp, tanto en pacientes normotolerantes, como en diabéticos tipo 2 de edades y grados de obesidad diferentes [18]. Además, ha demostrado capacidad predictiva en cuanto al desarrollo futuro de intolerancia a la glucosa y diabetes tipo 2 en estudios prospectivos [152, 153]. Todo ello hace que sea un buen método para estudios epidemiológicos amplios. Como inconveniente, refleja fundamentalmente la resistencia a nivel hepático que es la que predomina en ayunas y no la muscular [18].

Índice cuantitativo de control de sensibilidad a la insulina (QUICKI)

El QUICKI ("quantitative insulin sensitivity check index") es un índice de sensibilidad a la insulina que se calcula matemáticamente sumando los logaritmos de la glicemia e insulinemia basales; el resultado es el denominador de una fracción que tiene como numerador al 1, por tanto, si aumentan la glicemia e insulinemia como reflejo de resistencia a la insulina, el cociente (que es un índice de sensibilidad) desciende. Según los autores, la transformación logarítmica hace que los resultados correlacionen mejor con los del clamp [145].

Ventajas y limitaciones: Como un sustituto simple, útil, económico y mínimamente invasivo para las medidas de sensibilidad a la insulina derivadas del clamp, el QUICKI es apropiado y eficaz para su uso en estudios de investigación clínica, para hacer un seguimiento de los cambios después de las intervenciones terapéuticas, y para su uso en estudios donde la evaluación de la sensibilidad a la insulina no es de interés principal.

Índices derivados de pruebas dinámicas

Existen diversos indices que se calculan a partir del OGTT utilizando distintos protocolos para el muestreo [154], tales como el índice de Stumvoll [155], el índice de Aviñón [156], el índice de sensibilidad oral a la glucosa (OGSI) [157], el índice de Gutt [158], el índice de Belfiore, índices derivados del test de Matsuda entre otros [18].

El índice de sensibilidad de Matsuda [18] se basa en una fracción que tiene como denominador a la raíz cuadrada de un producto entre la glicemia e insulinemia basales e integradas a lo largo del OGTT y como numerador a la cifra 10.000; en su descripción, los autores reportan una correlación con los resultados del clamp superiores a los índices antes descritos y similar al HOMA.

Originalmente propuesto por Matsuda y DeFronzo, el índice de sensibilidad a la insulina Matsuda (ISI (Matsuda)) es un índice de sensibilidad a la insulina que refleja una estimación de la sensibilidad a la insulina hepática y muscular. Este índice se calcula a partir de las concentraciones de glucosa en plasma (mg / dl) e insulina (mIU / l) en ayunas y durante el OGTT.

La fórmula para el índice Matsuda -ISI es:

$$\mathrm{ISI}_{(\mathrm{Matsuda})} = \frac{10,000}{\sqrt{G_0 \times I_0 \times G_{\mathrm{mean}} \times I_{\mathrm{mean}}}}$$

G0 - concentración de glucosa plasmática en ayunas (mg / dl), I0 - concentración de insulina plasmática en ayunas (mIU / l), Gmean - concentración media de glucosa plasmática durante OGTT (mg / dl), de 0 a 120 min e Imean - concentración media de insulina en plasma durante OGTT (mIU / l), de 0 a 120 min.

Otro índice derivado del OGTT es el índice insulinogénico (IGI) [159]:

IGI = [30 min insulina insulina en ayunas (pmol / l)]

[30 minutos glucosa en ayunas glucosa (mmol / l)

El "disposition index" (DI) se usa para evaluar la función de las células beta, combinando tanto la secreción de insulina como la sensibilidad a la insulina.

DI = ISI × [AUC30 min insulina / AUC30 min glucosa], donde AUC30 min es el área bajo la curva entre la línea de base y 30 min de la OGTT para las mediciones de insulina (pmol / l) y glucosa (mmol / l), respectivamente), calculado por el método trapezoidal [160].

Para evaluar la resistencia a la insulina en el hígado y el músculo, existen dos índices, el índice de resistencia a la insulina hepática (HIRI) [18] y el índice de sensibilidad muscular a la insulina (MISI) siguiendo los métodos descritos por Matsuda y DeFronzo para HIRI [18] y Abdul-Ghani y otros para MISI [161].

2.7. Limitaciones de los métodos actuales de diagnóstico de diabetes mellitus tipo 2 y necesidad de nuevos biomarcadores

La diabetes tipo 2 suele permanecer varios años sin diagnosticar, debido a que la hiperglucemia se desarrolla gradualmente, y en etapas tempranas, no siendo lo suficientemente grave como para que el paciente perciba los síntomas clásicos de la enfermedad [148].

Los biomarcadores tradicionales utilizados para identificar pacientes con DMT2 y prediabetes incluyen parámetros relacionados con la homeostasis de la glucosa, especies lipídicas y scores como el FINDRISC [162] o el test de la ADA [29]. En 2010, la Asociación Americana de Diabetes (ADA) agregó la hemoglobina glicosilada (HbA1c) como criterio de diagnóstico para diabetes y prediabetes [3, 19], debido a la fuerte asociación con el alto riesgo de desarrollar la enfermedad. El uso de HbA1c como prueba diagnóstica tiene sus ventajas: menor variabilidad entre días, mayor estabilidad preanalítica y estandarización internacional. Además, el FINDRISC se ha implementado con éxito como una prueba de detección práctica para evaluar el riesgo de diabetes y detectar los casos no diagnosticados en poblaciones europeas [163, 164]. Sin embargo, estos parámetros y pruebas también tienen sus limitaciones y no pueden predecir con precisión el riesgo individual de desarrollar DMT2, principalmente porque ciertas condiciones médicas pueden afectar a la medida de HbA1c y causar falsos positivos o falsos negativos [165, 166]. Además, estudios previos han examinado el rendimiento de la HbA1c en comparación con la glucosa plasmática en ayunas (FPG) en el diagnóstico de la disglucemia en adultos mayores y los autores han demostrado una considerable discrepancia entre FPG y HbA1c en el diagnóstico de DMT2 y prediabetes, con diferencias acentuadas por raza y género [167, 168]. También se ha demostrado que la variabilidad en las determinaciones de HbA1c podría estar influenciada por parámetros que afecten a la glicación de la hemoglobina, incluyendo factores epidemiológicos, genéticos y fisiológicos [167].

Por lo tanto, los biomarcadores tradicionales disponibles hasta el momento (p. Ej. Glucosa plasmática en ayunas, HbA1c) (p. Ej., Adipoquinas, citocinas y proteína C reactiva) y scores como el test de la ADA y el FINDRISC, no son capaces de detectar eficazmente a los individuos con alto de desarrollar DMT2 porque identifican aquellos que ya presentan alteraciones metabólicas. Es necesaria la búsqueda de un nuevo modelo de diagnóstico precoz que mejore la sensibilidad,la especificidad y aumente el valor predictivo.

3. ENDOTOXEMIA COMO BIOMARCADOR DEL DESARROLLO DE DIABETES MELLITUS TIPO 2

3.1 Endotoxemia metabólica.

La microbiota intestinal está en contacto con la mucosa y, por lo tanto, afecta necesariamente al huésped y viceversa. Las bacterias asociadas a la mucosa están en contacto más directo con las células epiteliales intestinales (IEC) que con las demás bacterias que residen en el lumen, por ello, son muy importantes en la estimulación del sistema inmune del huésped, asi como en la integridad de la barrera intestinal [169]. La capa epitelial es la barrera final entre el contenido luminal y el huésped, por lo tanto, es esencial mantener su integridad, ya que un desequilibrio en ella se ha relacionado con el desarrollo de diversas enfermedades [170].

El término "integridad intestinal" se usa a menudo en conexión con la permeabilidad intestinal. Aunque estos dos términos están estrechamente relacionados, no son lo mismo. La integridad intestinal se define como "el mantenimiento de la barrera intestinal completa y ensamblada", que es posible gracias a la capa de moco, las IECs y la conexión entre las proteínas de unión cerrada o en inglés “Tight junction proteins” (TJs) [171]. La interacción entre IEC, a través de TJ, desmosomas, uniones de adhesión y uniones gap (**Figura 5**) es importante para mantener la integridad de la barrera intestinal. Por otro lado, la permeabilidad intestinal es un reflejo del paso de moléculas a través de la capa IEC y puede verse afectada tanto por las células epiteliales que forman la barrera, como por las interacciones por TJ.

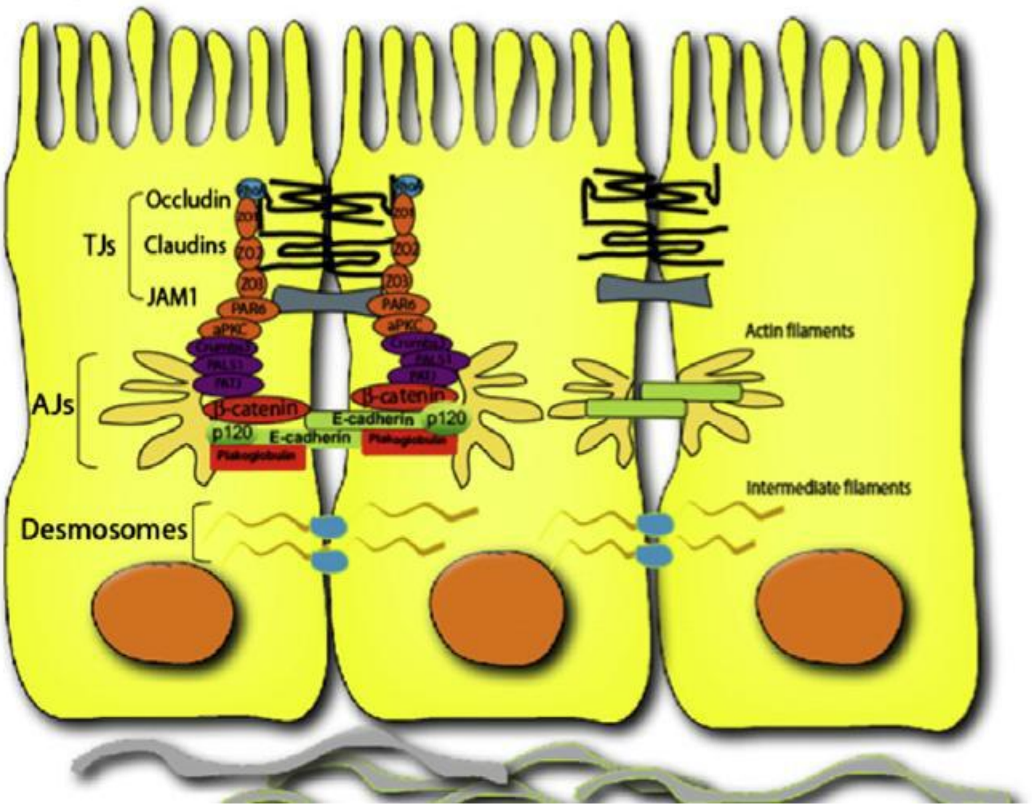

Figura. 5. Interacción entre células epiteliales a través de TJ, uniones adherentes y desmosomsa. Modificado de Hallstrand et al [172].

La alteración de la integridad intestinal o la permeabilidad intestinal permite el paso al torrente circulatorio de ciertas moléculas como el lipopolisacárido (LPS), lo que se ha relacionado con el desarrollo de enfermedades como el colon irritable, el síndrome metabólico, la obesidad y la DMT2 [173].

El LPS es un componente principal de la membrana externa de bacterias gramnegativas. Se considera una endotoxina, que contribuye a la integridad estructural y la protección de esa membrana contra el ataque químico. Está formado por lípidos y polisacáridos y tiene tres regiones estructural y genéticamente distintas: una porción llamada lípido A (responsable de la actividad endotóxica), un oligosacárido central y la región externa o antígeno O [174] (**Figura 6**).

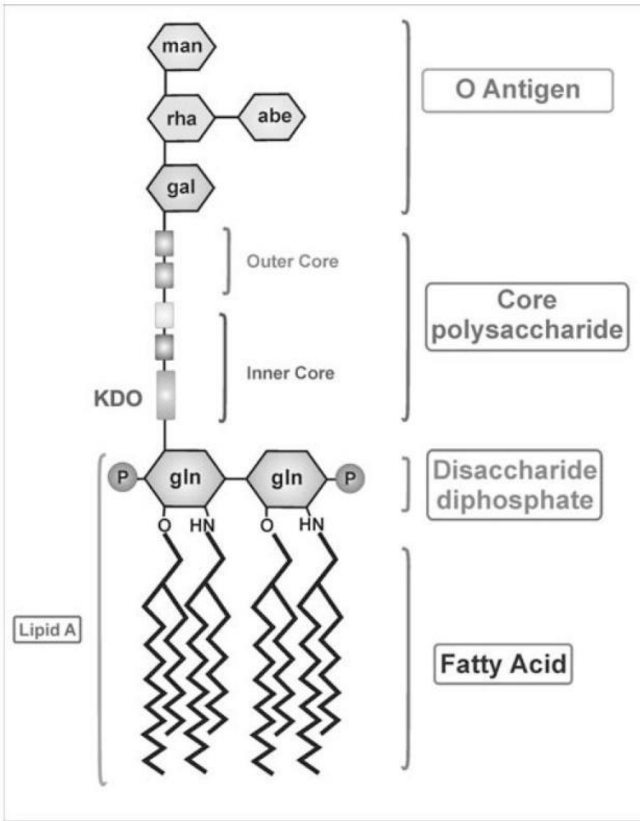

Figura 6. Estructura química del lipopolisacárido (LPS). Modificado de Galdiero et al [175].

Se han propuesto dos mecanismos de absorción de LPS [176]. Primero, en un estudio in vitro sobre un modelo de adenocarcinoma epitelial humano, se observó que la formación de quilomicrones promueve la absorción de LPS. El otro mecanismo sugerido consiste en la internalización del LPS por las células de los microconjuntos intestinales [176] y los enterocitos, con la participación del TLR4 (toll like receptor 4) y la proteína de diferenciación mieloide-2 (MD-2) [177] .

3.2 Endotoxemia y obesidad en el desarrollo de diabetes mellitus tipo 2

El aumento de peso se ha asociado con una mayor permeabilidad intestinal, así, estudios previos han demostrado que concentraciones altas de ácidos grasos alteran la integridad de la barrera intestinal [178] lo que promueve la absorción de LPS, aumentando sus niveles plasmáticos en dos o tres veces [179]. Como consecuencia, los niveles aumentados de LPS circulante conducen a una condición definida como "endotoxemia metabólica" [180] que puede desencadenar una respuesta inmune que induce inflamación. Es importante destacar que el intestino es la principal fuente de LPS, y se estima que la luz intestinal tiene más de 1 g de LPS. Solo una pequeña dosis de

endotoxina en el sistema circulatorio puede inducir activación de moleculas proinflamatorias [176] [181].
El punto de partida para la activación de la inmunidad innata es el reconocimiento de las estructuras conservadas de bacterias, virus y componentes fúngicos a través de receptores de reconocimiento de patrones (PRR)[182]. Los TLR son PRR que reconocen patrones moleculares asociados a microorganismos (MAMP) [183], así como varias estructuras bacterianas de membrana externa de las bacterias gramnegativas (LPS) y componentes de la pared celular grampositiva como el ácido lipoteicoico o peptidoglicano [182].

Por tanto, el reconocimiento de microorganismos conduce a la activación de la vía de señalización del factor nuclear kappa B (NF-κB) y, en consecuencia, desencadena la producción de citoquinas proinflamatorias y la regulación positiva de moléculas coestimuladoras en células presentadoras de antígenos, lo que lleva a la activación de células T [184].

Los tejidos diana para el LPS son el tejido adiposo, el hígado y el endotelio [185]. Por lo tanto, la porción responsable de la actividad endotóxica de LPS (lípido A) se une al TLR-4 presente en la membrana plasmática. El reconocimiento de LPS por este receptor está mediado por la proteína LBP, el correceptor CD14 del TLR-4 y por el MD-2 [186]. El TLR-4 se encuentra en la superficie de las células inmunitarias (monocitos, macrófagos, células de Kupffer) y las células no inmunes (adipocitos, hepatocitos y células endoteliales) (**Figura 7**). Por lo tanto, la endotoxemia esta relacionada con estados proinflamatorios como la obesidad suponiendo un factor de riesgo para el desarrollo de enfermedades como la DMT2 [187].

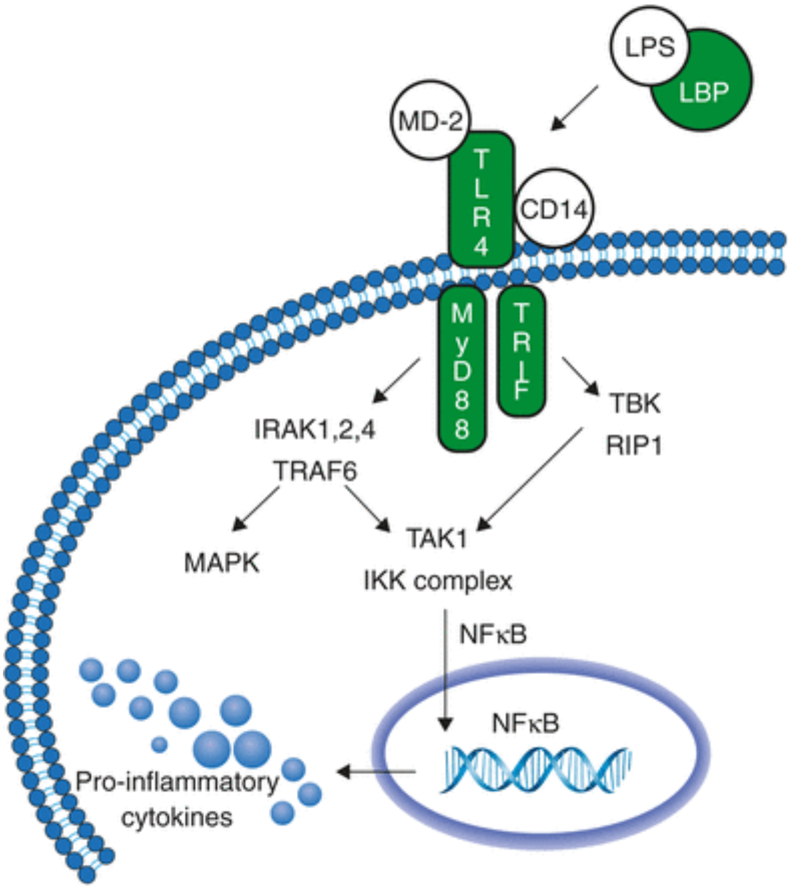

Figura 7. Vía de señalización LPS-TLR4. Modificado de Neves et al [188]

La importancia de la endotoxemia metabólica en la fisiopatología de la resistencia a la insulina y la obesidad ha sido destacada por Shi y colaboradores [189], quienes demostraron que los ratones que carecían de TLR4 estaban protegidos contra la resistencia a la insulina inducida por una dieta alta en grasas.

Estudios clínicos previos han demostrado que las concentraciones de LPS circulante y LBP son más altas en individuos con diabetes tipo 1 o 2, y en sujetos obesos [190-193], lo que fortalece su relación con la insulino resistencia y enfermedades metabólicas. Otros estudio también han demostrado que los niveles de LBP, proteína de unión a LPS, se incrementaron en la obesidad y el síndrome metabólico, y también se ha relacionado con la inflamación en adipocitos y la resistencia a la insulina [194, 195].

En un estudio realizado por Creeley y colaboradores [196] los individuos con DMT2 presentaron niveles de LPS más altos (76%) que los individuos sin la enfermedad. Los pacientes diabéticos fueron tratados con rosiglitazone y mostraron una reducción del 51% en los niveles de insulina y del 35% en niveles de LPS. La rosiglitazona es un agonista del PPAR-γ, el cual presenta propiedades antiinflamatorias [197]. Este antidiabético oral puede atenuar la inflamación, favoreciendo el aclaramiento de LPS, reduciendo la insulinemia y aumentando la biodisponibilidad de HDL. Las reducciones en las concentraciones de insulina en plasma favorecen la función de las células de Kupffer, que son en parte responsables del aclaramiento de LPS [196]. Los individuos con bajos niveles de HDL mostraron una mayor respuesta inflamatoria (TNF-α, IL-1beta, IL-6, IL-8) después del tratamiento con dosis bajas de LPS [198]. Además, Pajkrt y colaboradores demostraron que la infusión de HDL puede reducir los efectos nocivos de LPS [199].

4. MiRNAs BIOMARCADORES DE DESARROLLO DE DIABETES MELLITUS TIPO 2

4.1. Biogénesis y secreción de miRNAs

Los microARNs (miRNAs) son pequeñas moléculas de ARN no codificantes de 19-22 nucleótidos que regulan la expresión génica [200]. Desde su descubrimiento en 1993 por Ambros y colaboradores en *Caenorhabditis elegans*, se han identificado microRNAs en vertebrados, en plantas y en algunos virus [201]. Gracias a métodos computacionales y al uso de la bioinformática ha sido posible determinar su amplia distribución dentro del genoma humano, así como sus funciones en diferentes tejidos y en la regulación de la expresión de los genes. Actualmente, existe una gran cantidad de datos e información sobre miRNAs, prueba de ello son los miles de miRNAs maduros de *Homo sapiens* que han sido descritos y almacenadas en la base de datos miRBase [http://www.mirbase.org/]. Curiosamente, cada uno de estos microRNAs puede potencialmente regular la expresión de varios genes, mientras que un solo ARNm puede ser regulado por muchos miRNAs diferentes. Por lo tanto, no es sorprendente que los miRNAs estén implicados en la mayoría de los procesos biológicos y que su desregulación pueda dar lugar a diversas enfermedades, entre las que destaca la DMT2 y sus complicaciones [202]. Durante la última década, se ha visto que los miRNAs regulan genes implicados en la funcionalidad de las células beta pancreáticas, la secreción de insulina y la proliferación de la masa de células beta para compensar la resistencia a la insulina [203].

La biogénesis, maduración y secreción de los miRNAs constituyen mecanismos moleculares altamente complejos que aún no se conocen por completo.

Los miRNAs se procesan a partir de un precurson primario largo (pri-miRNA) que puede estar contenido en diferentes regiones génicas; intrónica, exónica, o en una región no traducida (UTR). La RNA polimerasa II se encarga de la transcripción del pri-miRNA, el cual, al igual que un ARNm, se encuentra poliadenilado y tiene un extremo CAP-5´ [204]. Cada pri-miRNA puede contener uno o más miRNAs, cada uno dentro de una secuencia de 60 a 80 nucleótidos que se encuentra plegada sobre sí misma para formar una estructura en forma de horquilla, la cual es referida como pre-miRNA [205]. Sin embargo, la mayoría de los pri-miRNAs están organizados en grupos o “clusters” dentro de una misma región cromosómica y presentan patrones de expresión similares, lo que sugiere que su transcripción se realiza en forma de policistrones que están regulados bajo un mismo promotor [206, 207]. A continuación, las horquillas de pre-miRNAs son reconocidas y escindidas dentro del núcleo celular por la acción de un complejo microprocesador que incluye a la enzima Drosha (RNAasa tipo III) [208] y su elemento asociado DGCR8 (proteína de la región crítica del gen 8 del síndrome DiGeorge) [209]. El complejo microprocessor escinde el pri-miRNA en un pre-miRNA de ~ 60-70 nucleótidos, creando una estructura imperfecta de stem-loop [208]. La eficiencia del reconocimiento y procesamiento del precursor de miRNA por Drosha depende del tamaño del bucle terminal, la estructura del tallo y las secuencias flanqueantes de los sitios de escisión de Drosha [208].

Una vez escindido, el pre-miRNa en forma de horquilla, contiene un segmento de dos nucleótidos en el extremo 3´ que es característico del corte producido por Drosha. Este rasgo permite al factor nuclear de exportación, conocido como exportina 5, reconocer al pre-miRNA y trasportarlo al citoplasma en un proceso dependiente de guanosina trifosfato (GTP) [210]. Una vez en el citoplasma, otro complejo formado por la RNasa tipo III, Dicer, y la proteía trans-activadora de unión a RNA (TRBP), realiza un segundo corte sobre el pre-miRNA para generar una molécula de RNA de doble cadena de 18 a 24 nucleótidos de longitud, que contiene dos potenciales miRNAs maduros. Este dúplex de ARN se asocia a un complejo proteínico denominado complejo silenciador inducido por ARN (RISC, RNA Induced Silencing Complex), en el que la proteína Argonauta 2 (Ago2) es el componente principal (**Figura 8**) [211].

Una de las dos cadenas de ARN es seleccionada por el RISC para actuar como “cadena guía” y constituir el miRNA maduro. La cadena restante, también denominada cadena “ pasajero” es degradada por la acción de la proteína Argonauta 2 (Ago 2). Parece ser, que la estabilidad termodinámica del extremo 5' de las cadenas que conforman el dúplex de miRNA maduro, determina la identidad de la cadena guía y la cadena pasajera [212].

Estudios previos han mostrado que el complejo RISC selecciona la cadena con menor estabilidad termodinámina al apareamiento con su ARNm blanco como la cadena guía al mismo tiempo que degrada a la cadena " pasajero", la más estable. Sin embargo, existen evidencias de que ambas cadenas del dúplex de ARN son susceptibles de ser reconocidas por RISC y coexistir en ciertos tejidos, para actuar sobre diferentes ARN mensajeros (ARNm) [213-215]. Después de la unión a RISC, el miRNA guiará este complejo catalítico a una secuencia específica del ARNm blanco, ejerciendo así una regulación postranscripcional de la expresión del gen diana [213-215].

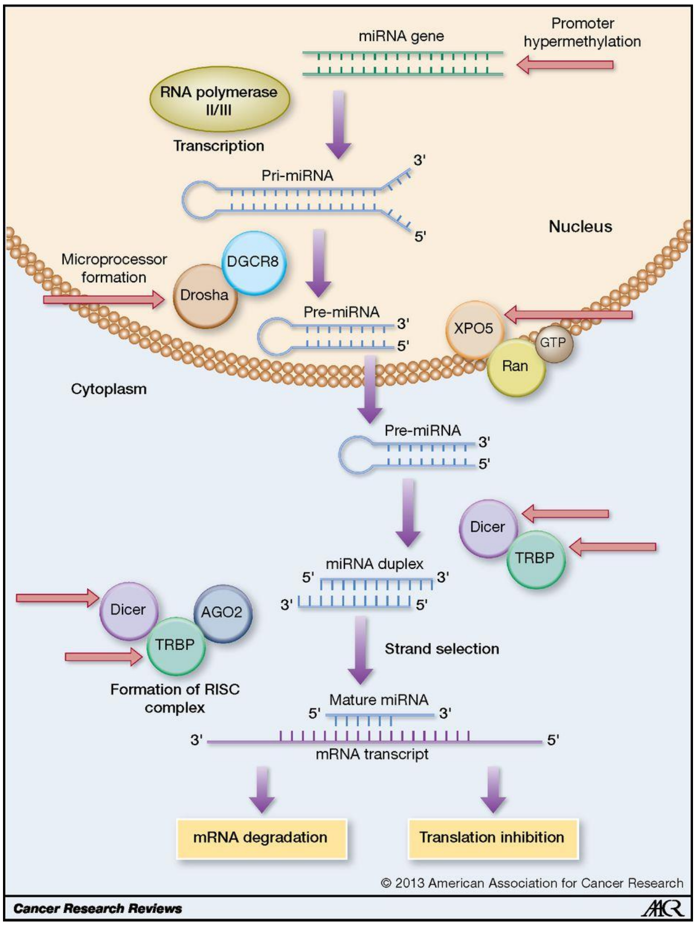

Figura 8: Biogénesis de miRNAs. Modificado de Mulrane et al [216]

Se han identificado distintas formas de secreción y transporte de los miRNAs (**Figura 9**):

1. Secreción activa de miRNAs dentro de exosomas derivados de Cuerpos Multivesiculares (MVB).

2. Generación de cuerpos apoptóticos: desprendimiento de microvesículas que contienen miRNA, tras una lesión tisular u otros estímulos.

3. Liberación pasiva a través de proteínas de unión a ARN (libres de vesículas).

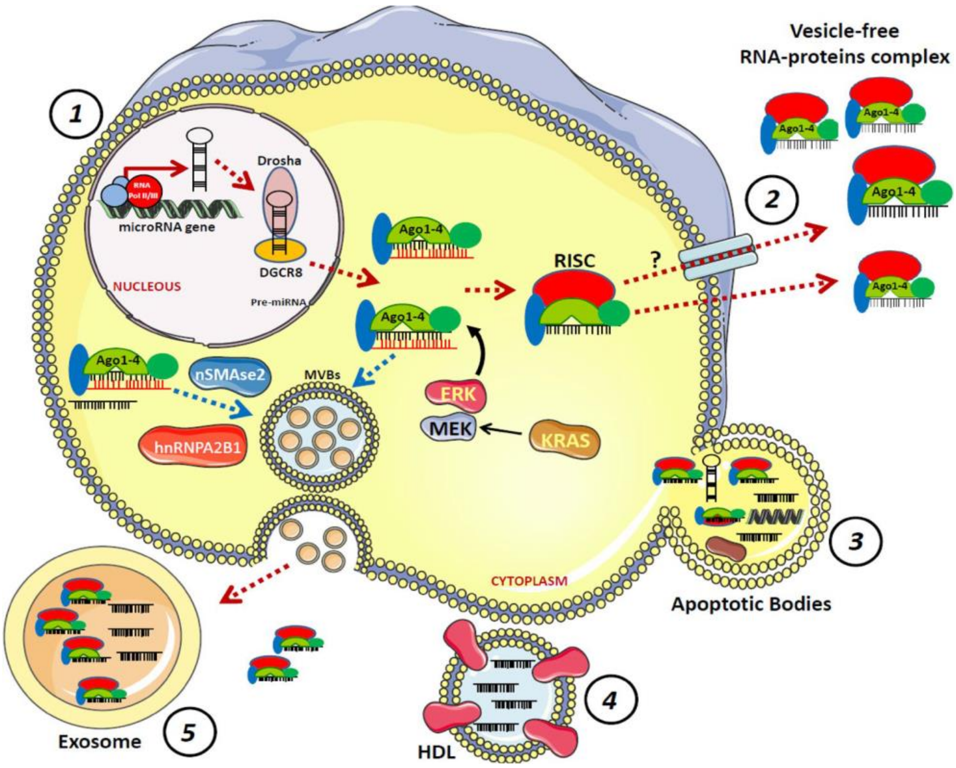

Figura 9: Mecanismos de secreción de los miRNAs circulantes. Modificado de Sebastiani et al [217]

Los miRNAs pueden secretarse activamente o liberarse pasivamente a través de diferentes mecanismos. **(1)** Los microRNAs son procesados a partir de un precursor primario largo conocido como pri-miRNA en un proceso secuencial de dos etapas guiado principalmente por dos enzimas, drosha en el núcleo y dicer en el citoplasma. **(2)** Los complejos de miRNA-proteínas (del complejo RISC) pueden liberarse a través de la secreción pasiva secundaria a la muerte celular; sin embargo, no está claro si existe un mecanismo de secreción activa involucrado en la liberación de estos complejos libres de vesículas. **(3)** La secreción pasiva de ARN puede también llevarse a cabo a través de cuerpos apoptóticos que contienen muchos componentes de celulares entre los que se incluye los complejos de miRNA-proteína. **(4)** La secreción activa incluye el acoplamiento de miRNAs con HDL. **(5)** Finalmente, los cuerpos multivisiculares (MVB), que, a su vez, maduran en exosomas o en otras clases de microvesículas, pueden cargarse específicamente con miRNAs o complejos de miRNAs-proteínas que dan como resultado una secreción activa y específica. La carga de miRNAs puede controlarse mediante varios mecanismos, incluida la actividad de nSMAse2 o hnRNPA2B1. Las vías de señalización que controlan la fosforilación de proteínas Ago2 (KRAS, MEK, ERK) pueden regular también la carga de complejos de miRNA-proteína en MVB y luego en exosomas.

Aunque no todos los mecanismos mencionados anteriormente son completamente conocidos, el más interesante es el de la secreción activa a través de exosomas. Los exosomas son pequeñas vesículas lipídicas (30-100 nm), liberadas por exocitosis cuando los MVB se fusionan con la membrana plasmática. La generación de exosomas cargados de miRNAs está regulada y estrechamente controlada por varios mecanismos. El mecanismo secretor dependiente de ceramida controlado por la esfingomielinasa 2 neutra (nSMAse2) es uno de ellos. Es de destacar que el silenciamiento del gen nSMAse2 conduce a una disminución de la secreción de miRNAs en exosomas y, a la inversa, su sobreexpresión induce un aumento de la tasa de secreción [218, 219]. Aunque el mecanismo exacto de carga de miRNAs en exosomas no se conoce completamente, se ha sugerido que puede ser un proceso rigurosamente controlado, seleccionando preferentemente microRNAs específicos. Este es el caso de las ribonucleoproteínas hnRNPA2B1, hnRNPA1 y hnRNPC. De hecho, la hnRNPA2B1 es capaz de reconocer la secuencia GGAG en el extremo 3 'del miRNA y cargar específicamente miRNAs con tales secuencias en exosomas [220].

Otro mecanismo de control de la carga de miRNAs en los exosomas es através de la proteína Ago2 de los complejos miRNA-proteínas. Se han publicado estudios sobre la presencia de complejos de Ago2-miRNA libres de vesículas en el espacio extracelular [221] o contenidos en exosomas [222]. Se ha demostrado que la activación de KRAS dependiente de MEK / ERK actúa específicamente sobre la fosforilación de Ago2, inhibiendo así la carga del complejo de Ago2-microRNAs en exosomas [223].

Los exosomas no son la única forma de transporte de miRNAs [224], también en complejos de proteínas libres de vesículas, tales como Ago2-microRNAs o NPM1-microRNAs [224] . Aunque se piensa principalmente que se trata de una liberación pasiva secundaria a la apoptosis celular, no podemos descartar la presencia de otras vías de secreción activa.

Además, recientemente se ha visto que los miRNAs pueden ser transportados junto con las HDL [225]. Finalmente, la liberación pasiva de miRNAs en cuerpos apoptóticos constituye otro mecanismo de transporte de estas pequeñas moléculas de ARN por los fluidos biológicos.

4.2 Mecanismo de acción de miRNAs

De manera general, los miRNAs establecen un apareamiento de bases con una secuencia de 2 a 8 nucleótidos que se localiza en la región 3´UTR del ARNm, denominada secuencia semilla. El apareamiento del miRNA con su ARNm blanco conduce a la degradación de este último o a la represión de su traducción [226].
El grado de complementariedad entre ambos, es lo que determina el mecanismo de regulación que se va a llevar a cabo. Si la complementariedad exhibida es perfecta, el miRNA induce la escisión del ARNm por acción del complejo RISC (suele ocurrir en plantas). Usualmente, la escisión ocurre dentro de la región de complementariedad miRNA-ARNm y requiere de la participación de Ago2 [227-229]. De manera alternativa, si la complementariedad es imperfecta, el RISC actúa silenciando o bloqueando la traducción del ARNm (represión traslacional), siendo este último, el mecanismo que ocurre con mayor frecuencia en mamíferos [230].

Este bloqueo no está del todo elucidado y parece llevarse a cabo durante la traducción, ya sea al inicio ó durante la etapa de elongación. Se ha demostrado que existe una represión del mecanismo de reconocimiento del CAP 5´, en el que el miRNA impide la unión del factor de inicio de la traducción el F4F [231].

Aquellos ARNm que han sido silenciados por los mecanismos mencionados anteriormente, son secuestrados dentro de un tipo de estructuras de procesamiento citoplasmáticas denominadas cuerpos P, en donde son almacenados y, finalmente, degradados por la acción de proteínas que también se encuentran dentro de los cuerpo P, tales como las enzimas que degradan el CAP 5´; Dcp1/Dcp2 y una exonucleasa; y las que eliminan progresivamente la poliadenilación en 3´como el complejo Ccr4p/Pop2p/Not. De hecho, todo el conjunto del RISC se encuentra dentro de estos cuerpos P, por lo que éste también puede ensamblar la maquinaria que conlleva al silenciamiento del ARNm [232]. Sin embargo, la represión traslacional no siempre es seguida por la degradación del ARNm, ya que se ha observado que un ARNm reprimido por un miRNA puede ser reactivado [233].

También se ha demostrado que, de manera opuesta, el miRNA puede dirigirse al extremo 5 'UTR [234]. La inclusión de codones raros puede inducir e incrementar la traducción del transcrito sobre el que ejercen su efecto, más que inhibir su expresión [235, 236].

4.3 miRNAs como biomarcadores de enfermedades

Además de su reconocida función reguladora intracelular, se sabe que los miRNAs pueden encontrarse en forma extracelular estable cirulante y ser detectados en los fluidos biológicos corporales tales como plasma, suero, orina, leche, saliva o humor acuoso [237, 238]. Los miRNAs extracelulares circulantes fueron descubiertos por primera vez en el plasma de la placenta materna [239]. En un estudio previo se analizaron 157 miRNAs diferentes y detectaron cuatro miRNAs placentarios altamente expresados en el plasma materno en una forma circulante. Este descubrimiento inició un campo de investigación complejo basado en la idea del uso de los microRNAs como biomarcadores de enfermedades, como lo demostraron posteriormente muchos otros estudios [240, 241].

Aunque diversos estudios se han enfocado en los mecanismos biológicos y los modos de transporte de los ARN extracelulares, en la actualidad, el papel exacto de los miRNAs circulantes aún no se ha dilucidado por completo. Sin embargo, se ha sugerido que los miRNAs pueden representar una nueva forma de comunicación intercelular. Dicha visión hipotética ha sido reforzada por el descubrimiento del transporte de miRNAs dentro de pequeñas vesículas lipídicas, tales como microvescículas y / o exosomas que los protege de la degradación por RNAsas y los hace más estables [242]. Varios estudios han demostrado que los miRNAs son liberados mediante la maquinaria secretora celular y son transferidos a los tejidos de destino donde pueden reanudar sus funciones [243, 244].

Por lo tanto, los miRNAs circulantes pueden reflejar la respuesta del organismo para intentar mantener la homeostasis o bien la progresión de diversas enfermedades. Así, los miRNAs se han propuesto como biomarcadores de diagnóstico y pronóstico para distintas enfermedades, como cáncer, trastornos neurológicos, enfermedades cardiovasculares, afecciones inflamatorias y la diabetes tipo 2 [245-250].

En comparación con los biomarcadores actualmente utilizados, el uso de miRNAs tiene varios beneficios, ya que se pueden medir de forma rápida y precisa gracias a las plataformas tecnológicas que se han desarrollado en los últimos años, son extradionariamente estables en comparación con los ARNm [251], pero, sobre todo, se expresan diferencialmente en diferentes tejidos y estados celulares, proporcionando una gran cantidad de información [252, 253].

Todo esto los hace extremedamente valiosos y útiles como biomarcadores de enfermedades. El aumento de los niveles séricos de varios miRNAs se ha relacionado con diferentes tipos de cáncer, por ejemplo, *miR-141* en cáncer de próstata [254], *miR-25* y *miR-223* en cáncer de pulmón [255]; *miR-21, miR-92, miR-93, miR -126 y miR-29a* en cáncer de ovario [256]; *miR-92 y miR-17-3p* en cáncer colorrectal [257].

Otros estudios mostraron como los miRNAs regulan la angiogénesis y la integridad del endotelio, ejerciendo un papel clave en la patogénesis de la aterosclerosis y la enfermedad cardiovascular o como biomarcadores de insuficiencia cardíaca e infarto agudo de miocardio. Por ejemplo, se ha visto que *miR-27b* y *miR-130a* estimulan la angiogénesis y *miR-221* y *miR-222* la inhiben [258]. El *miR-423-5p* se ha descrito como un marcador diagnóstico de insuficiencia cardíaca [259] y otros miRNAs como *miR-1, miR-133, miR-499 y miR-208* se encontraron elevados en sujetos tras un infarto agudo de miocardio [260].

4.4 Relación entre miRNAs y diabetes mellitus tipo 2

Desde el descubrimiento de los miRNAs, un número creciente de ellos se han encontrado involucrados en la patogénesis de la diabetes mellitus [261] y su desregulación puede conducir a un deterioro del metabolismo de la glucosa [262], la funcionalidad de la célula beta, la secreción de insulina y el desarrollo de la enfermedad (**Figura 10**). Las células beta pancreáticas desempeñan un papel central en la homeostasis de la glucosa a través de la secreción de insulina [263]. Se ha demostrado que los miRNAs regulan la supervivencia celular, la apoptosis, proliferación, diferenciación y funcionalidad de las células beta, especialmente la secreción de insulina [264].

Estudios previos han demostrado que los miRNAs regulan la expresión de genes proapoptóticos como *Bax* y antiapoptóticos como el gen *Bcl- 2* [265, 266] controlando así el proceso de apoptosis de célula beta. Se ha visto que en islotes de células beta de personas adultas, los niveles elevados de glucosa se acompañan de una reducción de los niveles de *miR-375* [267], induciendo la secreción de insulina por de-represión de sus dianas Mtpn [267] y PDK1 [268]. La sobreexpresión de *miR-375* atenúa la transcripción genética y producción de insulina, reduciendo la secreción de insulina inducida por glucosa [268, 269]. Además, el *miR-375* controla un conjunto de genes que regulan negativamente el crecimiento y proliferación celular [270], es así como la pérdida aberrante de este miRNA conduce a una reducción importante de la masa de células beta, conduciendo a niveles bajos de insulina, hiperglucemia y por tanto de diabetes. También se ha demostrado que el aumento en la expresión de *miR-29a/b* en modelos de animales diabéticos induce resistencia a la insulina [271].

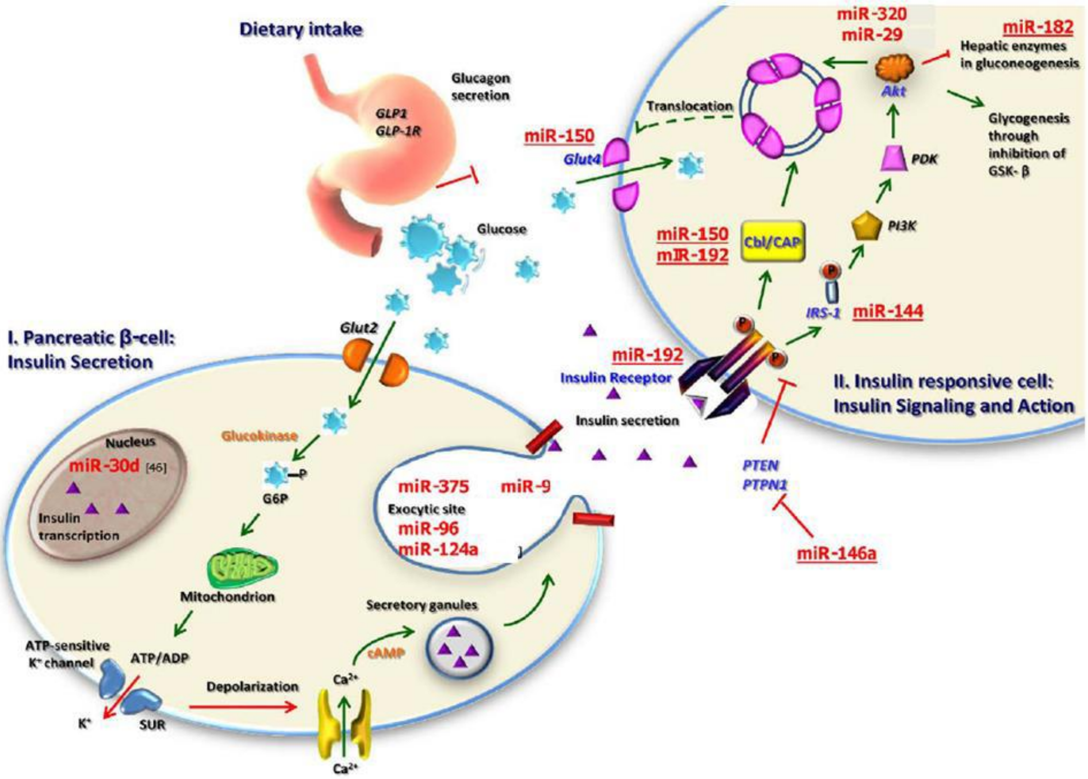

Figura 10: Posibles rutas metabólicas reguladas por miRNAs en las distintas etapas de la vía de señalización de insulina en la patogenesis de la DMT2. Modificado de Karolina, DS. et al [272]

Otros estudios han demostrado el papel de los miRNAs en la exocitosis de insulina en células beta. Por ejemplo, *miR-9* regula positivamente la secreción de insulina inducida por glucosa en las células beta inhibiendo el gen *Onecut-2*, el represor de granulofilina (synaptotagmin-like protein 4), una proteína que inhibe fuertemente la secreción de inducida por K+ [273]. Asimismo, *miR-96* también regula negativamente la exocitosis de insulina mediada por granulofilina y reduce los niveles de *Noc2*, lo cual perjudica la capacidad de la célula beta para responder a secretagogos [274]. Otro miRNA con influencia directa sobre la célula beta, es *miR-124a*, el cual se sobreexpresa en células beta en presencia de glucosa y además regula la expresión de proteínas relacionadas con la maquinaria de exocitosis [275]. Una acumulación excesiva de *miR-124a* en células beta conduce a una liberación exagerada de insulina a concentraciones de glucosa baja, pero a su vez, a una secreción de insulina reducida en respuesta a glucosa [275].

Un reciente estudio [272] comparó la expresión de miRNAs en modelos de ratas con diabetes y pre-diabetes, sin tratamientos farmacológicos, explorando las características diferenciales de los miRNA en diferentes órganos durante el desarrollo etiopatogénico de la DMT2, identificando los miRNA que pueden ser utilizados como marcadores para valorar el desarrollo de la enfermedad. Además, se identificaron ocho miRNAs importantes (*miR-144, miR-146a, miR-150, miR-182, 192 de miR, mir-29a, miR-30 d y miR-320*) que podrían participar en la regulación y señalización de insulina, así como ser útiles para distinguir diferentes etapas de progresión de la diabetes. Entre estos, el *miR-144* se ha descrito como un modulador directo de IRS1, gen que codifica para el receptor de insulina, y por lo tanto un objetivo terapéutico potencial de la DMT2 [272]. Además, se ha demostrado que los *miR-103/107* desempeñan un importante papel en la sensibilidad a la insulina y pueden representar nuevas dianas terapéuticas para el tratamiento de la DMT2 y obesidad [276].

Así, los miRNAs circulantes se han sugerido como biomarcadores para el diagnóstico de DMT2. Sin embargo, la mayoría de los estudios de miRNA relacionados con DMT2 hasta la fecha se llevaron a cabo en modelos de cultivos celulares, animales, o en grupos heterogéneos de pacientes con DMT2, con lo que no es posible excluir que hayan existido interferencias del fenotipo clínico y los medicamentos usados por los pacientes [255, 277-279].

Zampetaki y colaboradores [279] encontraron niveles circulantes disminuidos de los miRNAs (*miR-21, miR-24, miR-15a, miR-20b, miR-126, miR-191, miR-197, miR-223, miR-320 y miR-486*), en el plasma de pacientes con DMT2 (n=80) en comapración con un grupo control de sujetos no diabéticos (n=80). Basándose en los niveles circulantes de los cinco miRNAs más diferencialmente expresados, (*miR-15a, miR-320, miR-126,* *miR-223 y miR-28-3p*), fue posible identificar casi al 70% de los pacientes con diabétes, sin tener en cuenta otro parámetro.

Además, estudiaron si existían diferencias en los perfiles de expresión de miRNAs entre los sujetos normoglucémicos al inicio del estudio pero que desarrollaron diabetes tras un período de seguimiento de 10 años (casos incidentes, n=19) y un grupo control que no la desarrolló (n=19). Encontraron menores niveles en la situación basal de los miRNAs, *miR-15a, miR-29b, miR-126, miR-223* y mayores niveles del *miR-28-3p* en los casos incidentes de diabetes, lo que sugiere que los miRNAs podrían no sólo ser marcadores potenciales de diagnóstico de la DMT2, sino también ser utilizados para predecir el desarrollo de la enermedad.

Entre los miRNAs expresados diferencialmente entre sujeros diabéticos y no diabéticos, el *miR-126* (abundante en células endoteliales, contribuye al mantenimiento y la reparación de la integridad vascular y la angiogénesis) mostró una alta afinidad con procesos asociados con la DMT2 [280]. En un estudio posterior llevado a cabo por Zhang y colaboradores [281] en diabéticos, sujetos con glucosa alterada en ayunas (IFG) e individuos normoglucémicos, *miR-126* resultó ser el único con una expresión significativamente reducida en pacientes con IFG y DMT2 en comparación con los sujetos normoglucémicos, indenpendientemente de la edad y el sexo. Este estudio confirmó el papel de *miR-126* como biomarcador potencial para el diagnóstico y la predicción de la DMT2 [281]. Los mismos autores analizaron dos grupos de sujetos normoglucémicos, uno de los cuales desarrolló DMT2 tras 2 años de seguimiento, con el objetivo de confirmar el papel de los bajos niveles circulantes de *miR-126* como un marcador predictivo del desarrollo DMT2 en individuos susceptibles. Finalmente, demostraron que los niveles del *miR-126* en plasma habían disminuido antes de la aparición de DMT2 [282]. En un estudio transversal, Ortega y colaboradores analizaron el perfil de expresión de miRNAs circulantes en pacientes con DMT2 y en sujetos con tolerancia normal a la glucosa y observaron bajos niveles de *miR-126, miR-423-5p, miR-192, miR-195, miR-130b, miR-532 -5p, y miR-125b*, y altos niveles de *miR-140-5p, miR-142-3p y miR-222* en el grupo de pacientes con DMT2. En concreto, los niveles circulantes de los *miR-140-5p, miR-423-5p, miR-195 y miR-126* fueron capaces de discriminar a los pacientes con DMT2 del grupo control, con una precisión del 89.2% [283].

Además de *miR-126*, otros miRNAs circulantes se han identificado como candidatos a biomarcadores de DMT2. Se encontraron niveles significativamente elevados del *miR-146a* en el plasma de pacientes con diagnóstico reciente de DMT2 en comparación con sujetos con tolerancia normal a la glucosa, lo que sugiere un papel para este miRNA en la fisiopatología de la enfermedad [284]. Por el contrario, en un estudio realizado por Baldeón y colaboradores [285], el *miR-146a* mostró menores niveles en el suero de pacientes ecuatorianos con DMT2 en comparación con sujetos obesos y dislipémicos no diabéticos. Esta controversia podría ser debida a la diferente fuente de muestra (plasma o suero) o a diferencias en la población de estudio (origen étnico). Así, otro estudio llevado a cabo por Wang y colaboradores evaluó los niveles de 14 miRNAs en el plasma de 84 iraquíes (19 con DMT2) y 68 suecos (14 con DMT2). En toda la población, la expresión de *miR-24* y *miR-29b* fue significativamente diferente entre pacientes con DMT2 y no diabéticos, mientras que se observó una expresión más alta de *miR-144* en los pacientes diabétios suecos, pero no en iraquíes, lo que sugiere que la etnia podría estar interfiriendo [286].

Curiosamente, también se ha confirmado que existe una asociación entre el *miR-126* y la DMT2 en muestras de suero. Liu y colaboradores [287] examinaron la expresión de *miR-126* en muestras de suero de 160 pacientes con DMT2, 75 individuos con IFG, 82 con IGT y 138 con tolerancia normal a la glucosa. Los niveles séricos del *miR-126* fueron significativamente menores en pacientes con IGT, IFG y DMT2 que en sujetos sanos, y en pacientes con DMT2 con respecto a pacientes con IGT o IFG. Después de 6 meses de tratamiento (dieta y ejercicio en pacientes con IFG / IGT, insulina más dieta y ejercicio en pacientes con DMT2), los niveles de *miR-126* en suero incrementaron significativamente [287].

5. METABOLITOS COMO BIOMARCADORES DEL DESARROLLO DE DIABETES MELLITUS TIPO 2

5.1. Metabolómica dirigida vs no dirigida

Desde principios de la década de los noventa, ha ido emergiendo con fuerza la metabolómica, la última de las ciencias ómicas. Esta ciencia estudia el conjunto de metabolitos presentes en un sistema biológico, en particular, en biofluidos como orina, sangre, el fluido cerebroespinal, la saliva o incluso en tejidos. La metabolómica permite la cuantificación simultánea de miles de metabolitos diferentes dentro de una muestra dada utilizando metodologías sensibles y específicas, como la cromatografía de gases o líquidos acoplada a la espectrometría de masas. La ausencia o presencia de algunos de estos metabolitos, así como la concentración relativa entre ellos, puede ser un indicador de estados de enfermedad o de factores de predisposición a ella [288].

La metabolómica no dirigida se suele usar para comparar dos condiciones biológicas, por ejemplo, células tratadas con fármaco o modificadas genéticamente en comparación con células control. Como tal, la metabolómica no dirigida es más adecuada como una herramienta de descubrimiento para identificar metabolitos que cambian en respuesta a la manipulación de un sistema biológico (concentración relativa) en lugar de proporcionar la concentración exacta de un metabolito conocido (concentración real).

La metabolómica dirigida, que se centra en la medición de metabolitos conocidos agrupados en familias con estructuras químicas similares (por ejemplo, aminoácidos, acilcarnitinas, ácidos orgánicos, etc.), es una herramienta más cuantitativa, ya que a menudo implica el uso de metabolitos marcados con isótopos estables (generalmente 2H o 13C) como estándares internos para conocer la cantidad de un analito diana [289, 290]. Cuando se usan juntas (metabolómica dirigida y no dirigida), proporcionan una visión profunda y dinámica de las funciones metabólicas de las células, tejidos / órganos e incluso animales y humanos completos [291].

5.2 Plataformas analíticas en metabolómica y análisis de datos

La Resonancia Magnética Nuclear (RMN) y la espectrometría de masas de alta resolución (MS) son las principales plataformas analíticas usadas en metabolómica. La RMN tiene muchas ventajas, pero sus sensibilidad es relativamente baja en comparación con los métodos de MS, y las concentraciones de metabolitos pueden quedar por debajo del límite de detección. Cada técnica tiene ventajas y desventajas asociadas, y ninguna metodología analítica individual es ideal para todos los metabolitos. Una combinación de tecnología analítica y de separación avanzada y de alto rendimiento podría obtener una perspectiva amplia del metaboloma [292].

El perfil metabólico obtenido en una muestra biológica podría proporcionar una visión de los cambios en la abundancia de los metabolitos endógenos en respuesta a las perturbaciones de una enfermedad. Los análisis de componentes principales (PCA, Principal Component Analaysis) y el análisis discriminante de mínimos cuadrados parciales "supervisados" (PLS-DA, Partial Least Square Discriminan Analysis) proporcionan una evaluación exhaustiva de qué metabolitos cambian según las fases del desarrollo de enfermedad. El PCA score plot nos informa sobre la dispersión de los datos y muestra la posible presencia de outliers, grupos, similitudes y otros patrones. Así, los puntos situados muy lejos de la elipse central son valores atípicos, cuando se agrupan indican composiones metabolómicas similares y cuando se dispersan, un metaboloma diferente entre grupos de estudio. Los PLS-DA son análisis discriminantes dirigidos dónde se indica el grupo al que corresponde cada muestra. El VIP socre se obtiene tras los análisis de PLS-DA e indica la importancia de cada variable dentro del modelo para discriminar entre grupos. La calidad de los modelos obtenidos en los análisis de PCA y PLS-DA se evalúa según los parámetros de R^2 y Q^2. R^2, es una medida de la bondad del ajuste, es decir, indica lo bien que el modelo se ajusta a los datos. Un gran R^2 (lo más cerca de 1) es una condición necesaria para un buen modelo, pero no es suficiente, es decir, podemos tener modelos pobres (modelos que no pueden predecir), incluso con un gran R^2. Sin embargo, un valor bajo de R^2 indica mala reproducibilidad (mucho ruido) de los datos. Por su parte, Q^2 indica lo bien que el modelo predice nuevos datos. Un valor de Q^2>0.5 indica muy buena capacidad de predicción [293, 294].

5.3. Metabolitos como biomarcadores de enfermedades metabólicas

La sociedad humana moderna está plagada de una pandemia de enfermedades crónicas tales como la obesidad, la diabetes y las enfermedades cardiovasculares, en las que la desregulación metabólica desempeña un papel clave. El perfil de metabolitos o metabolómica, define el fenotipo químico de los seres vivos y, por tanto, tiene un potencial único para definir biomarcadores capaces de predecir el desarrollo de una enfermedad, evaluar su progresión, y para arrojar luz sobre las anomalías mecanicistas subyacentes [288].

Trabajos recientes han sugerido que la metabolómica desempeñará un papel cada vez más importante en la comprensión de las complejidades de la obesidad. La obesidad puede causar hiperlipidemia, enfermedades cardiovasculares, síndrome metabólico y enfermedad de hígado graso no alcohólico, entre otras. Un estudio reciente llevado a cabo en muestras de suero de personas obesas identificó metabolitos clave relacionados con la obesidad [295]. Los niveles de betaína, ácido piroglutámico, ácido pipecólico, N-fenilacetamida, ácido úrico, entre otros, mostraron un aumento significativo en sujetos obesos. Particularmente, la lisofosfatidilcolina 16 fue el metabolito más importante según el VIP score. En otro estudio publicado por Wang y colaboradores examinaron los cambios en los metabolitos plasmáticos entre adultos obesos y normopeso e identificaron un perfil de metabolitos capaces de discriminar entre ambos grupos. Observaron un aumento del 2-octenoilcarnitina, ácido eicosadienoico, tromboxano B2 y piridinolina y una disminución del glucosiduronato de vitamina D3, entre otros, en el grupo de obesos. Otro estudio informó que la biosíntesis de ácidos grasos, el metabolismo de fenilalanina, el metabolismo del propanoato y la degradación de valina, leucina e isoleucina se alteraron en la obesidad [296].

El propósito de un estudio reciente de Kim y colaboradores [297] fue examinar las diferencias en el perfil metabolómico entre hombres con sobrepeso / obesos y con peso normal, observando que los hombres con sobrepeso / obesidad mostraron niveles más altos de triglicéridos, colesterol total y colesterol LDL y niveles más bajos de colesterol HDL y adiponectina que los hombres delgados. Especialmente, identificaron tres liso-fosfatidilcolinas como posibles marcadores plasmáticos para hombres con sobrepeso / obesidad. Con el uso de las plataformas analísticas basadas en RMN, se encontró que los lípidos séricos, los lípidos insaturados, la glucoproteína, el mioinositol, el piruvato, la treonina, la tirosina y la creatina se encontraron más elevados en obesos, mientras que la glucosa sérica y la urea estaban más bajas en obesos [298].

La metabolómica también se aplicó para predecir nuevos eventos cardiovasculares [299]. Por ejemplo, en el estudio CATHGEN, se identificó un metabolito formado por acilcarnitinas dicarboxiladas de cadena corta a media que predijo la muerte por infarto de miocardio. Además, la metabolómica también se ha utilizado para revelar un vínculo entre la dieta, la microbiota intestinal, el metabolismo del huésped y biomarcadores de eventos cardiovasculares [300]. Asi, por LC-MS se detectaron 18 analistos asociados con eventos cardiovasculares, tres de ellos relacionados con el metabolismo de la colina (colina, betaína y N-óxido de trimetilamina (TMAO)).

5.4. Metabolómica aplicada a la diabetes mellitus tipo 2

A diferencia de los genes o las proteínas, los metabolitos son biomarcadores de la actividad bioquímica y están estrechamente relacionados con los fenotipos clínicos [301], siendo capaces de revelar alteraciones en mecanismos biológicos que subyacen a las enfermedades [302].

Varios estudios han confirmado la asociación entre la metabolómica y la DMT2. Así, estudios previos han identificado un grupo de metabolitos compuestos por aminoácidos de cadena ramificada (BCAA, del inglés branched-chain amino acids) aminoácidos aromáticos (Phe y Tyr) y acilcarnitinas que se asociaron fuertemente con la resistencia a la insulina medida por los valores de HOMA-IR. En cierto sentido, este hallazgo fue simplemente un redescubrimiento, dado que la asociación de BCAA y aminoácidos aromáticos con la obesidad y la resistencia a la insulina se informó hace más de 40 años por Felig y colaboradores (1969) [302]. En concreto, la asociación de los BCAA con la resistencia a la insulina fue más fuerte que la observada para varios grupos de metabolitos lipídicos [290]. Esta asociación se confirmó en múltiples estudios [303-305] con grandes cohortes. Así, en 7.098 jóvenes finlandeses (edad media 31 años), se identificaron 39 metabolitos, entre los que destacan BCAA, aminoácidos aromáticos, cetonas y ácidos grasos, que mostraron una alta correlación con la resistencia a la insulina medida por el HOMA-IR [306]. De manera similar, en 2.204 mujeres de la cohorte Twins se encontró una fuerte asociación de BCAA y sus metabolitos con la DMT2 y niveles alterados de glucosa en ayunas [307].

Los estudios de metabolómica dirigida y no dirigida han revelado que los BCAA y sus metabolitos son biomarcadores predictivos del desarrollo de DMT2. Así, los niveles plasmáticos elevados de Leu, Ile, Val, Phe y Tyr se asociaron con un riesgo 5 veces mayor de desarrollar DMT2 en las cohortes de los estudios Framingham y Malmo [300]. Sin embargo, otros metabolitos, como el α-hidroxibutirato, linoleoil-glicerofosfocolina y ácido oleico, se asociaron más fuertemente con un estado de resistencia a la insulina en comparación con BCAA o glicina, y además se correlacionaron selectivamente con tolerancia alterada a la glucosa en múltiples cohortes [308, 309]. Varios grupos también han demostrado que los BCAA y sus metabolitos relacionados están asociados con la enfermedad coronaria [310, 311].

Más recientemente, la metabolómica no dirigida ha ampliado el rango de metabolitos que predicen el riesgo de desarrollar DMT2, entre los que se encuentran los lípidos. Así, el perfil lipidómico completo de los mismos 189 diabéticos y 189 sujetos no diabéticos de la cohorte de Framingham reveló que una asociación entre mayor riesgo de desarrollar DMT2 y triglicéridos ricos en ácidos grasos de cadena corta con un alto número de dobles enlaces, mientras un bajo riesgo se asoció con el contenido en TG ricos en ácidos grasos de cadena larga con bajo número de dobles enlaces [312].

Además, se identificó el ácido 2-aminoadípico (2-AAA) como metabolito fuertemente correlacionado con DMT2 [313]. En un metanálisis reciente de ocho estudios prospectivos, varios aminoácidos sanguíneos (leucina, valina, tirosina y fenilalanina) se asociaron positivamente con el riesgo de DMT2, mientras que otros (glicina y glutamina) se asociaron negativamente con el riesgo de enfermedad [314].

III. HYPOTHESIS

III. HYPOTHESIS

Type 2 diabetes mellitus (T2DM) is a major public health problem and its prevalence has increased over the past few decades, with no signs of receding in the near future [30]. In addition, patients with acute myocardial infarction (AMI) and T2DM have a considerably higher risk of developing a new cardiovascular event than those without T2DM [315]. The early identification of patients with a higher risk of developing T2DM is therefore very important to prevent the onset of the disease and efficiently control the associated cardiovascular risk factors. The traditional biomarkers used to identify T2DM and pre-DM patients include glucose homeostasis related parameters, lipid classes, and scores such as the Finnish Diabetes Risk Score (FINDRISC) [162]. In addition, the American Diabetes Association (ADA) added in 2010 the glycated hemoglobin (HbA1c) as a diagnostic criterion for diabetes and prediabetes [3, 19]. However, these parameters and tests also have their limitations and cannot precisely predict an individual's risk of developing T2DM.

Circulating miRNAs in human biofluids such as blood [247] has led to their use as non-invasive biomarkers for multiple pathologies including cardiovascular disease and T2DM [316-318]. There is evidence to suggest that a specific profile of circulating miRNAs could become a valuable biomarker to identify those normoglycemic and prediabetic individuals at increased risk of developing T2DM [279, 319, 320]. Moreover, a previous study has shown the association between endotoxemia and the risk of developing diabetes [321]. However, the relationship observed was subjected to several uncontrolled confounding factors [22, 322]. Unlike genes or proteins, metabolites are biomarkers of the biochemical activity and previous studies have confirmed the association between the metabolites profile and the new-onset T2DM. Considering the potential usefulness of miRNAs, metabolite profile and endotoxemia, until now, no studies aimed at testing this idea have been carried out in a large population at high risk of developing T2DM.

Based on these findings, our hypothesis is that plasma levels of different biological markers, such as miRNAs, LPS and metabolite profile, could become highly valuable and useful as predictive biomarkers to identify normoglycemic and prediabetic subjects with a higher risk of developing T2DM.

IV. OBJECTIVES

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Main Objective

To identify new biomarkers with the potential to evaluate the probability and predict the develop of type 2 diabetes mellitus in high-risk patients with coronary heart disease.

This general objective is divided into three objectives, which correspond to the three papers contained in this thesis:

1. To evaluate whether longitudinal fasting and postprandial measures of LPS and LPS binding protein (LBP) may improve the prediction of T2DM incidence, in the framework of the CORDIOPREV study. *This objective is addressed in paper 1 "Postprandial endotoxemia may influence the development of type 2 diabetes mellitus".*
2. To identify whether circulating miRNAs, added to clinical and biochemical markers (HbA1c, glucose, insulin, glucose after a 2 h oral glucose tolerance test [OGTT], hepatic insulin resistance index [HIRI], insulinogenic index [IGI], insulin sensitivity index [ISI], disposition index [DI], muscle insulin sensitivity index [MISI], homeostatic model assessment-insulin resistance [HOMA-IR] and FINDRISC), would improve the potential to predict new incident cases of T2DM. *This objective is addressed in paper 2 "Circulating miRNAs as Predictive Biomarkers of Type 2 Diabetes Mellitus Development in Coronary Heart Disease Patients: from the CORDIOPREV Study".*
3. To study whether plasma circulating levels of miRNAs according to T2DM or pre-DM status could be used as biomarkers to evaluate the risk of developing the disease. *This objective is addressed in paper 3 "A plasma circulating miRNAs profile predicts Type 2 Diabetes mellitus and prediabetes: from the CORDIOPREV study".*

Secondary objective:

To evaluate the differences in the metabolomics profiles between subjects who develop and those who not develop T2DM after 3 years of follow-up in order to identify metabolites with potential value as predictive biomarkers of T2DM.

1. Postprandial endotoxemia may influence the development of type 2 diabetes mellitus

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Randomized Control Trials

Postprandial endotoxemia may influence the development of type 2 diabetes mellitus: From the CORDIOPREV study

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SUMMARY

Background & aims: Insulin resistance (IR) and impaired beta-cell function are key determinants of type 2 diabetes mellitus (T2DM). Intestinal absorption of bacterial components activates the toll-like receptors inducing inflammation, and this in turn IR. We evaluated the role of endotoxemia in promoting inflammation-induced insulin resistance (IR) in the development of T2DM, and its usefulness as predictive biomarker.

Methods: We included in this study 462 patients from the CORDIOPREV study without T2DM at baseline. Of these, 107 patients developed T2DM according to the American Diabetes Association (ADA) diagnosis criteria after a median follow-up of 60 months (Incident-DIAB group), whereas 355 patients did not developed it during this period of time (Non-DIAB group).

Results: We observed a postprandial increase in lipopolysaccharides (LPS) levels in the Incident-DIAB at baseline ($P < 0.001$), whereas LPS levels were not modified in the Non-DIAB. Disease-free survival curves based on the LPS postprandial fold change improved T2DM Risk Assessment as compared with the previously described FINDRISC score (hazard ratio of 2.076, 95% CI 1.149–3.750 *vs.* 1.384, 95% CI 0.740–2.589). Moreover, disease-free survival curves combining the LPS postprandial fold change and FINDRISC score together showed a hazard ratio of 3.835 (95% CI 1.323–11.114), linked to high values of both parameters.

Conclusion: Our results suggest that a high postprandial endotoxemia precedes the development of T2DM. Our results also showed the potential use of LPS plasma levels as a biomarker predictor of T2DM development.

Clinical trials.gov.identifier: NCT00924937.

Abbreviations: IR, Insulin resistance; LPS, lipopolysaccharides; ADA, American Diabetes Association; LBP, LPS binding protein; CORDIOPREV, Coronary Diet Intervention with Olive Oil and Cardiovascular Prevention study; CHD, coronary heart disease; MED, Mediterranean diet; LF, low-fat diet; ISI, Insulin sensitivity index; IGI, insulinogenic index; Incident-DIAB, non-diabetic patients at baseline who developed T2DM after a median of 60 months of follow-up; Non-DIAB, non-diabetic patients at baseline who did not develop T2DM after the follow-up period; AUC, area under the curve.

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1. Introduction

Insulin resistance (IR) and impaired beta-cell function are key determinants of T2DM, although the underlying mechanisms and the precise temporal sequence vary extensively among populations [1]. In addition to the undisputed role that IR plays in the pathogenesis and prediction of the disease [2], it is also a therapeutic target once hyperglycemia is present [3]. Specifically, skeletal muscle IR is considered to be the initiating or primary defect that may be evident decades before b-cell failure and overt hyperglycemia develops [4].

Obesity is a major contributor to IR [5] and it has been associated to the chronic activation of inflammatory pathways causally linked to IR [6]. However, despite the association between obesity and T2DM, not all obese individuals develop T2DM, notwithstanding the fact that most patients with T2DM in Western countries are obese [7].

The molecular mechanisms responsible for activating inflammatory pathways in obesity are not well understood, but differences in phenotypic flexibility may condition the presence or absence of metabolic disorders such as IR and T2DM. Thus, it has been proposed that the capacity to timely respond and adapt to changing exposures may make the difference between remaining healthy or developing the disease [8,9].To evaluate this phenotypic flexibility, the body's homeostasis must be disrupted by tests such as a fat challenge or a glucose overload, and the responses measured using appropriate biomarkers [8,9].

In addition to the environmental changes, the organism is subjected to internal challenges such as those potentially posed by dysbiosis of the microbiota. Intestinal absorption of bacterial components such as the endotoxin lipopolysaccharides (LPS), bacterial DNA and flagellins, are known to activate the toll-like receptors, inducing inflammation, which may promotes IR [10]. However, this sequence of events is not well-established, and it is still unclear whether IR precedes the changes in the microbiota or vice-versa [11]. Moreover, several studies have shown that changes in the gut microbiota trigger the pathogenic mechanisms to promote the development of obesity, T2DM and metabolic syndrome [12].

A previous study has shown the association between endotoxemia and the risk of developing diabetes [13]. However, in this study, LPS was measured in a variable postprandial period (with a median time of 5 h an interquartile range of 3–7 h), after the ingestion of a non-standardized meal with dissimilar fat and carbohydrate contents (which has been reported to affect the postprandial LPS levels [14]). Furthermore, the fat content was not adjusted by the individual's body weight and T2DM diagnosis was not performed according to the American Diabetes Association (ADA) diagnosis criteria [15]. Therefore, the relationship observed between endotoxemia and T2DM incidence was subjected to several uncontrolled confounding factors. In order to shed light on the role of endotoxemia in promoting inflammation-induced IR leading to the development of T2DM, we investigated whether longitudinal fasting and postprandial measures of LPS and LPS binding protein (LBP) may improve the prediction of T2DM incidence, in the framework of the CORDIOPREV study. In addition, the inclusion in our experimental design of a postprandial fat challenge (mixed meal), which generates metabolic stress [16,17], may help classify individuals according to their phenotypic flexibility.

2. Material and methods

2.1. Study subjects

The Coronary Diet Intervention with Olive Oil and Cardiovascular Prevention study (CORDIOPREV; Clinical Trials.gov.Identifier: NCT00924937) is an ongoing prospective, randomized, open, controlled trial of 1002 patients receiving conventional treatment for coronary heart disease (CHD) who had their last coronary event over 6 months before enrollment in one of two different dietary models [a Mediterranean (MED) diet and a low-fat (LF) diet] over a period of 7 years. The patients were recruited between November 2009 and February 2012, mostly at the Reina Sofia University Hospital, Cordoba, Spain. The eligibility criteria, design and methods of the CORDIOPREV clinical trial have been reported elsewhere [18]. Briefly, patients were eligible if they were aged between 20 and 75 years, had established CHD without clinical events in the last 6 months, were thought to follow a long-term dietary intervention and did not have severe diseases or an estimated life expectancy of less than 5 years. All patients gave their written informed consent to participate in the study. The trial protocol and all amendments were approved by the local ethic committees, following the Helsinki declaration and good clinical practices. The experimental protocol conformed to international ethical standards.

We included in present work the 462 patients who had not been clinically diagnosed with T2DM at baseline in the CORDIOPREV-DIAB [19]. However, 79 were impair fasting glucose (IFG), 81 were impair glucose tolerance (IGT), 53 were both IFG and IGT, 180 had HbA1c in the range 5.7–6.4%, and 69 had no alteration in glucose metabolism at baseline. From a total of these 462 non T2DM patients at the beginning, 107 patients developed T2DM, according to the ADA diagnosis criteria [15], after a median of follow-up of 60 months. Baseline characteristics of the subjects in the study are shown in Supplemental Table 1.

2.2. Study design

The study design has been previously described [18]. Briefly, participants were randomized to receive two diets: a MED diet or a LF diet. The LF diet consisted of <30% total fat (<10% saturated fat, 12%–14% MUFA fat, and 6–8% PUFA fat), 15% protein, and a minimum of 55% carbohydrates. The MED diet comprised a minimum 35% of calories as fat (22% MUFA fat, 6% PUFA fat, and <10% saturated fat), 15% proteins, and a maximum of 50% carbohydrates. In both diets, the cholesterol content was adjusted to <300 mg/d.

2.3. Methodology of the two metabolic challenges

Two metabolic challenges, a fat overload and an oral glucose tolerance test, were performed on consecutive days at the beginning of the study and after 3 years of follow-up. Before starting the test, the patients had fasted (food/drugs) for 12 h and were asked to refrain from smoking during the fasting period and from alcohol intake during the preceding 7 days. They were also asked to avoid strenuous physical activity the day before the test was given. Details are provided as Supplemental Materials and Methods.

2.4. Dietary assessment

At the beginning of the study and every year, each patient had a face-to-face interview with a nutritionist to fill in a 137-item semi-quantitative food frequency questionnaire, validated in Spain [20], and well as a validated 14-item questionnaire of adherence to the Mediterranean diet to produce a Mediterranean diet score [21]. MED and LF diets were designed to provide a wide variety of foods, including vegetables, fruit, cereals, potatoes, legumes, dairy products, meat and fish. Participants in both intervention groups received the same intensive dietary counseling. Nutritionists administered personalized individual interviews at inclusion and every 6 months, and quarterly group education sessions were held

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with up to 20 participants per session and separate sessions for each group.

2.5. Clinical plasma parameters

Venous blood was collected in tubes containing EDTA at the times indicated above and used to analyze the participants' biochemical variables. Lipid variables, serum insulin and plasma glucose were determined as previously reported [19]. Insulin sensitivity index (ISI), HOMA-IR, insulinogenic index (IGI) and disposition indexes were calculated as previously described [19].

2.6. Measurement of plasma biomarkers

The endotoxin lipopolysaccharide (LPS) was measured in all the 462 patients at baseline and at 3 years of follow-up using the limulus amebocyte lysate test (QCL-1000 Chromogenic LAL (Lonzalberica S.A., Spain), as previously described [22]. LPS Binding Protein (LBP) levels were determined using a human LBP ELISA kit (HycultBiotech, Netherlands). Plasma concentrations of LBP, IL-6, MCP1 and TNF-a were measured in a subpopulation of 226 patients at baseline and at 3 years of follow-up (98 Incident-DIAB, 78 of which have already developed T2DM after 3 years of follow-up, and 128 Non-DIAB). Plasma levels of IL-6, MCP1 and TNF-a were determined using the Human IL-6 Quantikine HS ELISA Kit, Human CCL2/MCP-1 Quantikine ELISA Kit, and Human TNF-alpha/TNFSF1A Quantikine HS ELISA Kit (R&D Systems, Inc.).

2.7. Statistical analysis

All data presented are expressed as mean ± SEM. PASW statistical software, version 20.0 (IBM Inc., Chicago, IL, USA) was used for statistical analysis of the data. The normal distribution of variables was assessed using the Kolmogorov–Smirnov test. When values did not follow a normal distribution, they were $\log_{10}$ transformed. P values $\leq$ 0.05 were considered statistically significant. Details are provided as Supplemental Materials and Methods.

3. Results

3.1. Baseline characteristics of the participants

We observed that the values of BMI, weight, waist circumference, serum triacylglycerols (TAG), HbA1c, glucose, insulin and HOMA-IR were higher and the ISI, IGI and disposition index values were lower in the Incident-DIAB than in the Non-DIAB group (all, $P < 0.05$) (Table 1).

3.2. Influence of endotoxemia in T2DM development

To investigate the influence of endotoxemia in the development of T2DM, we measured the LPS and LBP plasma levels in the fasting state and 4 h after the intake of the mixed meal at the beginning of the study (Fig. 1). At baseline, no differences were found in fasting LPS plasma levels between groups; however, we did observe a postprandial increase in LPS plasma levels after the intake of the mixed meal in the Incident-DIAB group ($P < 0.001$), while no changes were observed in the Non-DIAB group (Fig. 2). When we analyzed the LBP plasma levels, we did not observe significant differences between the groups at baseline (Fig. 3).

Moreover, we also measured LPS and LBP plasma levels after the intake of a mixed meal administered at 3 years of follow-up between the 78 out of the 107 Incident-DIAB that had already developed T2DM at 3 years of follow-up and the Non-DIAB group. LBP plasma levels (fasting and postprandial measurements together) were higher in the Incident-DIAB group than in the Non-DIAB group ($P = 0.005$) (Fig. 3).

Finally, we investigated whether diet had an effect on the observed changes in endotoxemia in our population, as patients in each group were receiving a MED diet or a LF diet. No significant differences in LPS or LBP plasma levels were found after the consumption of LF or MED diets in the entire population or in the Incident-DIAB or Non-DIAB groups separately (data not shown).

3.3. Development of postprandial lipemia and T2DM

In order to assess whether differences in the postprandial TAG levels, as measured by the chylomicron formation rate, may determine the postprandial absorption of LPS, we evaluated TAG plasma levels after the intake of the mixed meal administered at baseline and at 3 years of follow-up. We observed higher postprandial TAG levels in the Incident-DIAB group than in the Non-DIAB group at baseline after 2, 3 and 4 h following the consumption of the mixed meal ($P = 0.045$, $P = 0.028$ and $P = 0.012$, respectively).

When we analyzed the postprandial TAG levels at 3 years of follow-up between the 78 out of the 107 Incident-DIAB that had already developed T2DM at 3 years of follow-up and the Non-DIAB group, we observed higher TAG plasma levels after 3 and 4 h of the intake of the mixed meal (statistical trend, $P = 0.092$ and $P = 0.052$, respectively). Moreover, we analyzed the AUC of TAG plasma levels and we observed higher levels in the 78 out of the 107 Incident-DIAB patients that had already developed T2DM at 3 years of follow-up than in the Non-DIAB group ($P = 0.028$) (Fig. 2).

3.4. Plasma levels of inflammatory cytokines in T2DM development

In order to assess the inflammatory status before and after T2DM development, we measured plasma levels of the inflammatory cytokines IL6, TNF-a, and MCP1. Whereas no differences between groups were observed at baseline, after 3 years of follow-up, we observed higher TNF-a levels in the 78 out of the 107 Incident-DIAB patients that had already developed T2DM at 3 years of follow-up than in the Non-DIAB group (fasting and postprandial values together) ($P = 0.032$) (Fig. 3).

Table 1
Baseline characteristics of the population for type 2 diabetes mellitus incidence study. Means values ± S.E.M. Incident-DIAB: patients who developed T2DM but were non-diabetic at baseline. Non-DIAB: non-diabetic patients. BMI: body mass index. HbA1c: glycated hemoglobin A1c. ISI: insulin sensitivity index. IGI: insulinogenic index. One-way ANOVA P-values.

	Incident-DIAB	Non-DIAB	P-value
n	107	355	n/a
Men/women (n)	87/20	302/53	n/a
Age (years)	58.75 ± 0.87	57.33 ± 0.50	0.171
Weight (kg)	85.70 ± 1.47	82.49 ± 0.72	0.037
BMI (kg/m^2)	31.39 ± 0.47	29.88 ± 0.22	0.002
Waist circumference (cm)	105.28 ± 1.08	101.73 ± 0.57	0.003
Serum triacylglycerols (mg/dL)	132.60 ± 6.60	119.45 ± 3.24	0.059
Total cholesterol (mg/dL)	164.97 ± 3.41	160.65 ± 1.62	0.217
HDL-cholesterol (mg/dL)	43.52 ± 1.04	44.58 ± 0.53	0.355
LDL-cholesterol (mg/dL)	93.40 ± 2.66	91.10 ± 1.33	0.421
CRP (mg/L)	2.88 ± 0.29	2.51 ± 0.17	0.329
HbA1c (%)	6.03 ± 0.03	5.86 ± 0.02	<0.001
HbA1c (mmol/mol)	42.37 ± 0.36	40.51 ± 0.19	<0.001
Fasting glucose (mg/dL)	96.18 ± 1.04	92.59 ± 0.53	0.002
Fasting insulin (mU/L)	10.51 ± 0.66	8.34 ± 0.31	0.001
ISI	3.35 ± 0.20	4.32 ± 0.14	0.001
HOMA-IR	3.37 ± 0.30	2.58 ± 0.09	0.001
IGI	0.64 ± 0.30	1.08 ± 0.06	0.025
Disposition index	0.83 ± 0.05	1.03 ± 0.03	0.003

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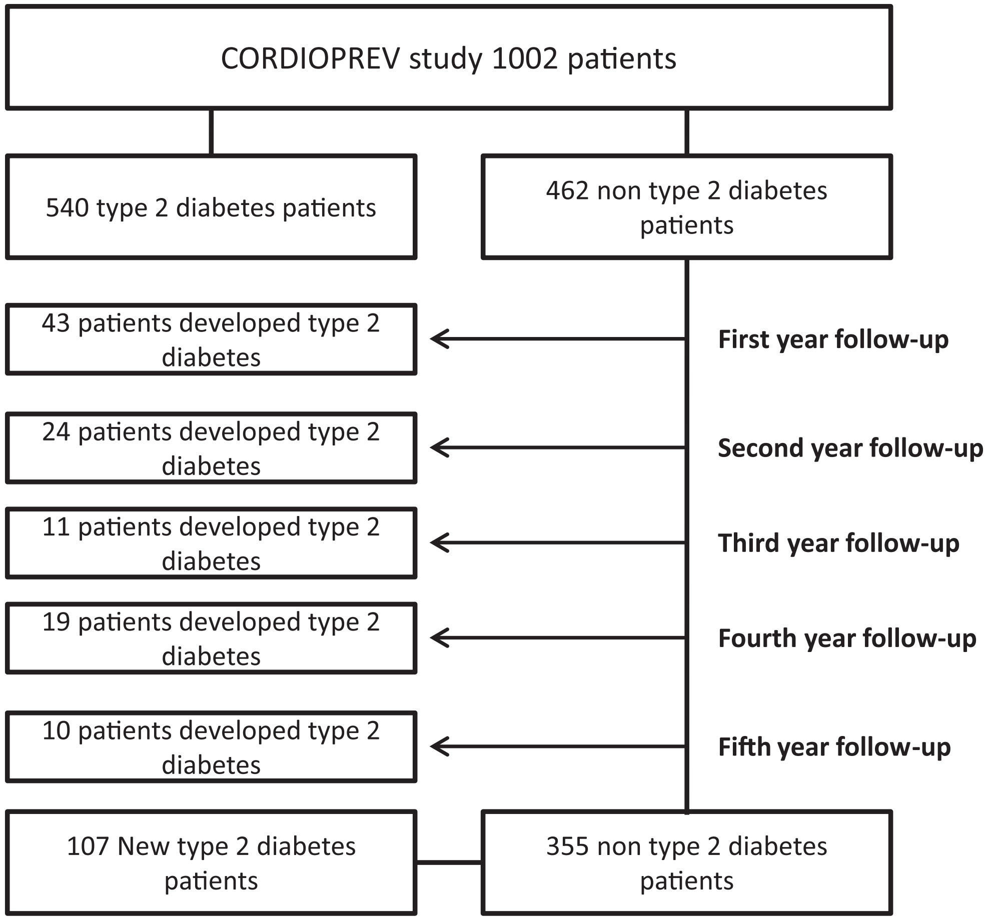

Fig. 1. Scheme of the study design.

We also analyzed the IL6 and MCP1 plasma levels, but no differences were found between groups and no effect was observed after the development of T2DM (Supplemental Fig. 1). In addition, when we analyzed the effects of diet on inflammatory cytokines, we observed no statistical differences between diets (data not shown).

3.5. Diabetes-free survival analysis

We performed a Cox proportional hazards regression analysis in order to determine the potential use of LPS postprandial levels as an independent predictor of T2DM development. To do that, the fold change between the postprandial and fasting LPS plasma levels was calculated and categorized by tertiles (ascending order). Once performed, the analysis was adjusted by age, gender, diet, BMI, HDL-c, AUC of TAG plasma levels, HbA1c and ISI index. We observed a hazard ratio (HR) of 1.752 and 2.074 (95% CI 0.966–3.177 and 1.147–3.747, respectively) between the patients with lower LPS postprandial increase (tertile 1) and patients with intermediate (tertile2) and high (tertile 3) postprandial increase, respectively. In addition, we also categorized patients by HbA1c tertiles (ascending order), and we calculated the Cox proportional hazards regression analysis adjusted by age, gender, diet, BMI, HDL-c, and ISI index, obtaining an HR of 1.264 and 3.120 (95% CI 0.706–2.263 and 1.828–5.326, respectively) between the patients with lower HbA1c levels (tertile 1) and patients with intermediate (tertile 2) and high (tertile 3) HbA1c levels, respectively. Moreover, we also categorized the AUC of TAG by tertiles (ascending order), and we calculated the Cox proportional hazards regression analysis adjusted by age, gender, diet, BMI, HDL-c, HbA1c and ISI index, obtaining an HR of 1.452 and 1.556 (95% CI 0.802–2.628 and 0.853–2.840, respectively) between the patients with lower AUC of TAG (tertile 1) and patients with intermediate (tertile 2) and high (tertile 3) AUC of TAG, respectively. When we performed the Cox proportional hazards regression with the FINDRISC Score [23], an index that identifies individuals at high risk for T2DM, adjusted by diet, HDL-c and AUC of TAG, HbA1c and ISI index, we observed an HR of 1.519 and 1.673 (95% CI 0.860–2.683 and 0.934–2.998, respectively) between the patients with lower FINDRISC Score (tertile 1) and patients with intermediate (tertile 2) and high (tertile 3) FINDRISC Score, respectively (Fig. 4).

In addition, we combined the LPS postprandial fold change, HbA1c levels and the AUC of TAG with the FINDRISC score, dividing the latter by the median, which produced the following groups: group 1, with low FINDRISC (lower than median) and low LPS (tertile 1); group 2, with low FINDRISC (lower than median) and intermediate-high LPS (tertiles 2 and 3); group 3, with high FINDRISC (higher than median) and low LPS (tertile 1); and group 4, with high FINDRISC (higher than median) and intermediate-high LPS (tertiles 2 and 3). Cox proportional hazards regression were calculated for the LPS postprandial fold change combined with the FINDRISC, and adjusted by age, gender, diet, BMI, HDL-c, AUC of TAG plasma levels, HbA1c and ISI index. The addition of the LPS postprandial fold change to the FINDRISC score to assess the risk of T2DM development increased to an HR of 3.977 (95% CI 1.372–11.527) between the patients with lower FINDRISC score and low LPS postprandial fold change, and patients with a high FINDRISC score and intermediate-high LPS postprandial fold change. We followed the same strategy, combining the HbA1c levels with

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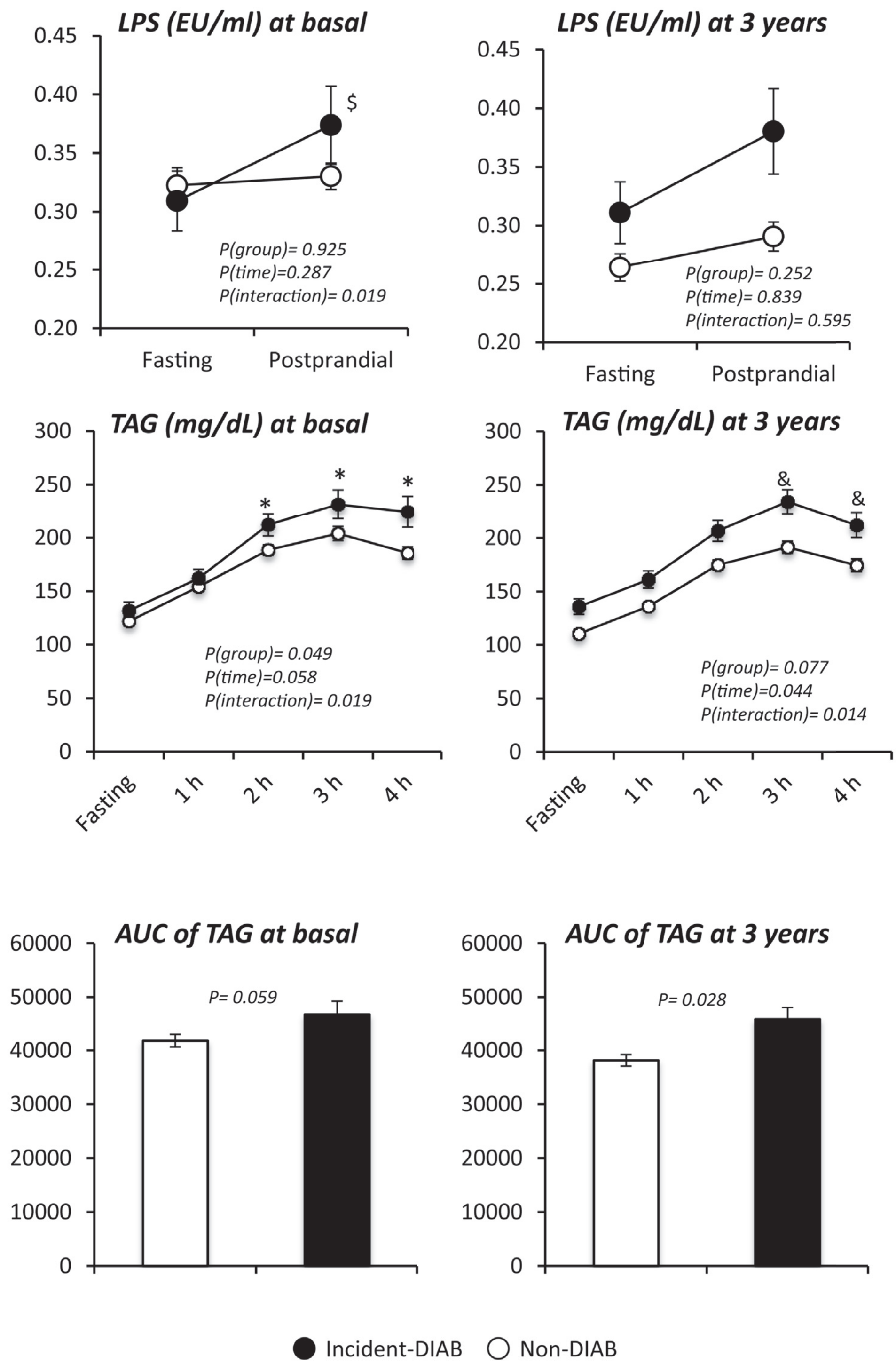


Fig. 2. Fasting and postprandial LPS and TAG circulating levels. Mean (±S.E.M.) of LPS (EU/mL) and TAG (mg/dL) plasma levels at 12-h fasting and after the administration of the mixed meal. Left panel, baseline: Incident-DIAB, patients who developed T2DM after a median of follow-up of 60 months as compared with Non-DIAB group, patients who did not develop T2DM after the follow-up period. Right panel, 3 years of follow-up: comparison between the Incident-DIAB patients who had already developed T2DM at this time (78 out of 107 patients) and the Non-DIAB group. AUC: area under the curve calculated by the trapezoidal method of TAG levels after the administration of the mixed meal at baseline and at 3 years; univariate analysis of AUC (area under curve) with age, gender and BMI as co-variables was used to determine the statistical differences between groups. LPS and TAG levels were analyzed by ANOVA for repeated measures *P*-values adjusted by age, gender and BMI. $^{\$}P < 0.05$ between postprandial and fasting in the Post-Hoc Bonferroni's multiple comparison tests. $^{*}P < 0.05$ and $^{\&}P < 0.1$ between groups in the Post-Hoc Bonferroni's multiple comparison tests. LPS, TAG, and AUC values were log transformed before statistical analysis.

the FINDRISC score, and we observed an HR of 2.890 (95% CI 1.456–5.737) between the patients with lower FINDRISC score and low HbA1c levels and patients with high FINDRISC and intermediate-high HbA1c levels. Moreover, combining the AUC of TAG with the FINDRISC score, and we observed an HR of 2.102 (95% CI 0.886–4.984) between the patients with lower FINDRISC score and low AUC of TAG and patients with high FINDRISC and intermediate-high AUC of TAG (Fig. 5).

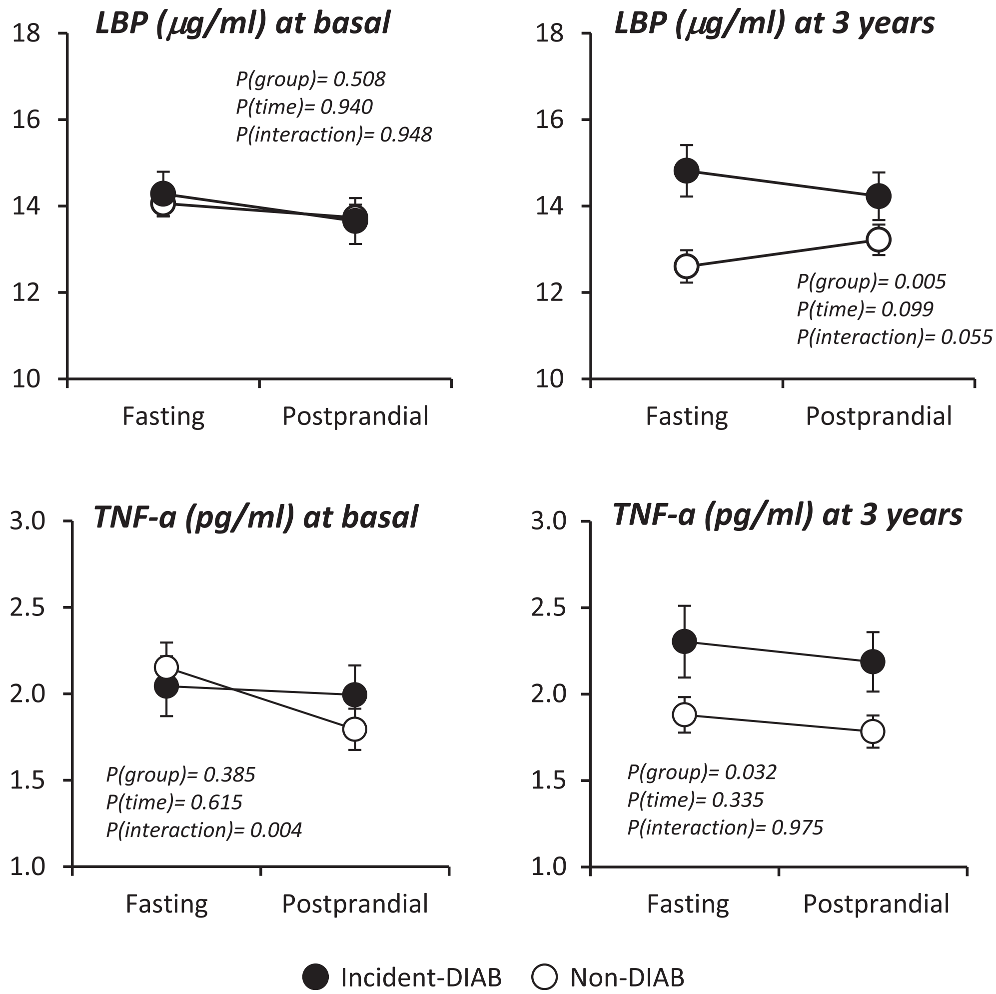


Fig. 3. Fasting and postprandial levels of LBP and TNF-a. Mean (±S.E.M.) of LBP (µg/mL) and TNF-a (pg/mL) plasma levels at 12-h fasting and after the administration of the mixed meal. Left panel, baseline: Incident-DIAB, patients who developed T2DM after a median of follow-up of 60 months as compared with Non-DIAB group, patients who did not develop T2DM after the follow-up period. Right panel, 3 years of follow-up: comparison between the Incident-DIAB patients who had already developed T2DM at this time (78 out of 107 patients) and the Non-DIAB group. ANOVA for repeated measures *P*-values adjusted by age, gender and BMI. TNF-a values were log transformed before statistical analysis.

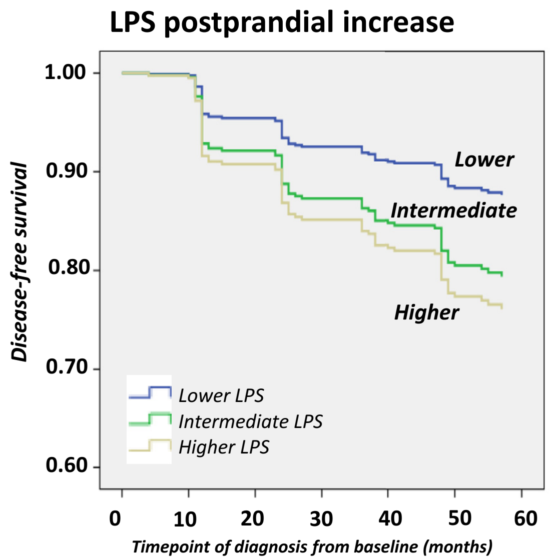
4. Discussion

Our data show a significant increase in baseline postprandial plasma levels of LPS and TAG following a mixed meal, among subjects with incident T2DM, as compared to those who remained T2DM-free after a median of follow-up of 60 months. These differences in LPS and TAG were not observed in the fasting state, which supports the usefulness of a metabolic challenge to discriminate among individuals with different phenotypic flexibility, which in turn may predict the presence or absence of metabolic disorders such as IR and T2DM [8,9].

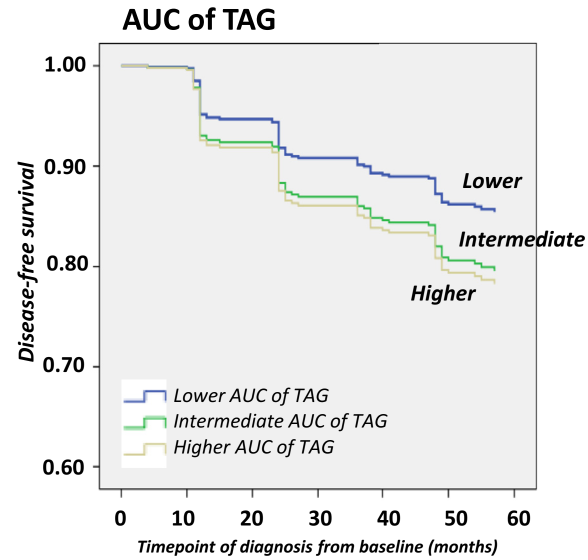
Our intestines harbor a vast microbial community [24], which acts collectively as an organ that is fully integrated in host metabolism and is involved in energy extraction from nutrients, regulates innate and adaptive immunity and is involved in energy balance [25]. It has been depicted that the gut lumen contains more than 1 g of LPS, a component of the Gram (-) bacterial cell wall) [26]. LPS can enter systemic circulation via two major routes: the paracellular route, through tight junctions between intestinal epithelial cells (IEC) [27]; and the transcellular route, through epithelial cells [28] as a result of raft recruitment of LPS-related signaling proteins leading to signaling and endocytosis [29]. In addition, a transcellular mechanism involving LPS internalization by IEC through the apical surface and LPS transport to the Golgi, to be further incorporated into chylomicrons, has been proposed [30]. The latter would help explain the postprandial inflammatory response observed after food intake, which is closely associated with the postprandial increase of TAG in plasma [17].

Our results suggest that a higher postprandial TAG increase in patients who developed T2DM may facilitate higher LPS absorption through the transcellular mechanism, as chylomicron formation promotes intestinal absorption of lipopolysaccharides [30]. The fact that these differences were observed at baseline suggests that higher postprandial endotoxemia precedes the classical diagnosis of T2DM by years. Moreover, the higher LPS levels observed in the study performed at 3 years of follow-up (when 78 out of the 107 Incident-DIAB had already developed T2DM), supports the notion that once diabetes is established, there is additional impairment of the integrity of the intestinal barrier. Thus, it is likely that, at this time point, LPS is absorbed by the paracellular route through tight junctions in addition to the fat-induced LPS absorption involving chylomicron formation.

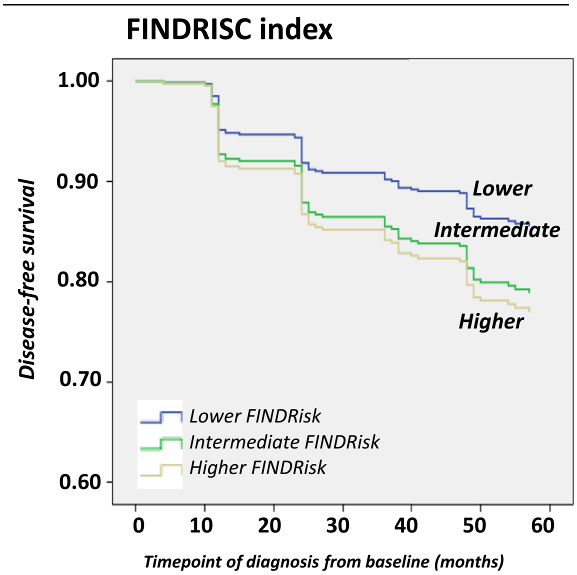
Furthermore, these changes in the intestinal barrier integrity may be associated with changes in the gut microbiota, which appears to be linked with pathogenic mechanisms that promote the development of obesity and T2DM [12,31], inasmuch as changes in



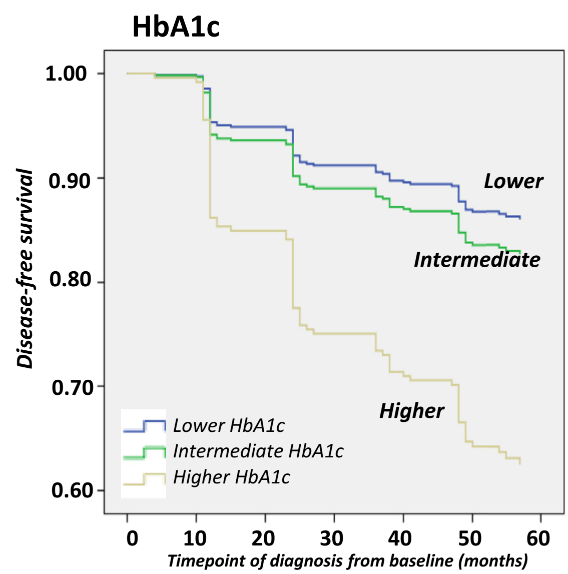
	Exp(B)	95.0% IC for Exp(B)	
		Lower	Upper
FC_LPS_basal T1-T2	1.752	0,966	3.177
FC_LPS_basal T1-T3	2.074	1.147	3.747



	Exp(B)	95.0% IC for Exp(B)	
		Lower	Upper
AUC_TAG_basal T1-T2	1.452	0.802	2.628
AUC_TAG_basal T1-T3	1.556	0.853	2.840



	Exp(B)	95.0% IC for Exp(B)	
		Lower	Upper
FINDRISC_basal T1-T2	1.519	0.860	2.683
FINDRISC_basal T1-T3	1.673	0.934	2.998



	Exp(B)	95.0% IC for Exp(B)	
		Lower	Upper
HbA1c_basal T1-T2	1.264	0.706	2.263
HbA1c_basal T1-T3	3.120	1.828	5.326

Fig. 4. Disease-free survival by COX proportional hazards regression analysis according to the postprandial increase in LPS after the fat challenge. The fold change between postprandial and fasting LPS plasma levels at baseline was calculated and patients were categorized by tertiles (ascending order). The LPS model was adjusted by age, gender, diet, BMI, HDL-c, AUC of TAG and HbA1c and ISI index, the AUC TAG model was adjusted by age, gender, diet, BMI, HDL-c and HbA1c and ISI index, and the HbA1c model was adjusted by age, gender, diet, BMI, HDL-c and ISI index. FINDRISC model was not adjusted to avoid over-fitting. The hazard ratio (HR) between tertile 1 and tertile 2; and between expression tertile 1 and tertile 3 was calculated. T1: tertile 1; T2: tertile 2; T3: tertile 3.

gut microbiota composition have been shown to affect intestinal permeability [32,33]. This idea is supported by the fact that plasma levels of LBP, an acute phase protein that responds to invasive bacterial infection [34], were higher among those patients who had developed T2DM, possibly as consequence of changes in the gut microbiota composition.

Initially, low levels of LBP were thought to potentiate cell responses to LPS whereas high LBP concentrations were inhibitory [35]. Further studies have shown that high LBP levels appear to be associated with an increased risk of bacterial infection and immune and hemodynamic derangement, rather than suppression of these responses [36]. The latter is also supported by our study, which

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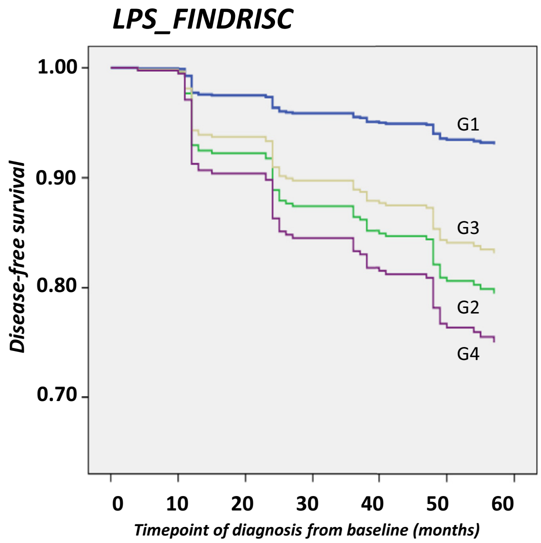


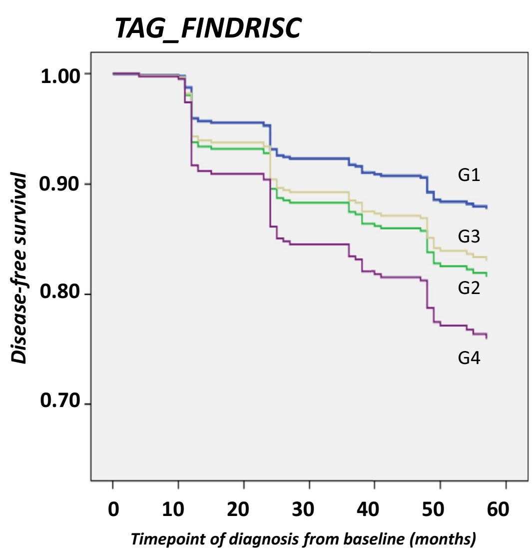
	Exp(B)	*95.0% IC for Exp(B)*	
		Lower	*Upper*
LPS_FINDRISC G1-G2	3.181	1.097	9.222
LPS_FINDRISC G1-G3	2.558	0.811	8.066
LPS_FINDRISC G1-G4	3.977	1.372	11.527

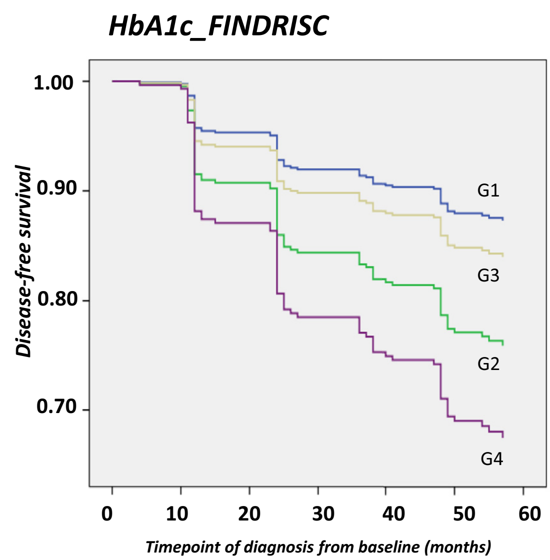
Group 1: Low FINDRISC-Low LPS

Group 2: Low FINDRISC-Intermediate/High LPS

Group 3: High FINDRISC-Low LPS

Group 4: High FINDRISC-Intermediate/High LPS





	Exp(B)	*95.0% IC for Exp(B)*	
		Lower	*Upper*
TAG_FINDRISC G1-G2	1.555	0.654	3.701
TAG_FINDRISC G1-G3	1.419	0.529	3.811
TAG_FINDRISC G1-G4	2.102	0.886	4.984

	Exp(B)	*95.0% IC for Exp(B)*	
		Lower	*Upper*
HbA1c_FINDRISC G1-G2	2.028	0.984	4.177
HbA1c_FINDRISC G1-G3	1.283	0.604	2.728
HbA1c_FINDRISC G1-G4	2.891	1.456	5.737

Group 1: Low FINDRISC-Low TAG

Group 2: Low FINDRISC-Intermediate/High TAG

Group 3: High FINDRISC-Low TAG

Group 4: High FINDRISC-Intermediate/High TAG

Group 1: Low FINDRISC-Low HbA1c

Group 2: Low FINDRISC-Intermediate/High HbA1c

Group 3: High FINDRISC-Low HbA1c

Group 4: High FINDRISC-Intermediate/High HbA1c

Fig. 5. Disease-free survival by COX proportional hazards regression analysis according to the postprandial increase in LPS and TAG after the fat challenge in combination with FINDRISC. Patients were categorized by the fold change in LPS plasma levels between the postprandial and fasting periods at baseline into tertiles (ascending order) and by the FINDRISC score divided by the median. Patients were categorized in the following groups: group 1, with low FINDRISC (lower than median) and low LPS (tertile 1); group 2, with low FINDRISC (lower than median) and intermediate-high LPS (tertile 2 and 3); group 3, with high FINDRISC (higher than median) and low LPS (tertile 1); and group 4, with high FINDRISC (higher than median) and intermediate-high LPS (tertile 2 and 3). LPS postprandial fold change combined with the FINDRISC model was adjusted by age, gender, diet, BMI, HDL-c, AUC of TAG plasma levels, HbA1c and ISI index. The same was performed combining the AUC of TAG and HbA1c with FINDRISC score, and adjusted by age, gender, diet, BMI, HDL-c, HbA1c (only in AUC of TAG model), and ISI index.

showed an increase in TNF-a plasma levels in parallel with the LBP increase. Based on this, LBP plasma levels may serve as a dysbiosis marker during the development of T2DM.

In summary, our results show that high postprandial triglyceridemia-associated endotoxemia precedes the diagnosis of diabetes by years. This idea is also supported by our disease-free survival analysis, which shows that patients with a low LPS postprandial increase are at a low risk of disease as compared with those categorized as having an intermediate or high LPS postprandial increase. Moreover, compared with a previous study in the

FINDRISC97 cohort, which associated endotoxemia and T2DM development, our study showed a higher predictive value, even taking into account that our study population was smaller (462 in CORDIOPREV, hazard ratio of 2.074, 95% CI 1.156–3.619 *vs*. 7169 subjects in FINDRISC97, 1.596, 95% CI 0.870–2.927 [13]). This difference in the prediction may lie in the fact that we administered a standard mixed meal, measured LPS before and 4 h after the meal, and used the LPS fold change (defined as the postprandial/fasting ratio) as inputs for T2DM prediction. By contrast, in the study performed with the FINDRISC97 cohort, LPS was measured within a variable time range after the ingestion of a meal. In fact, participants in the FINDRISC97 study were asked to fast 4 h before blood extraction (5 h as a median time of blood sampling after meal ingestion, with an interquartile range of 3–7 h), which may have resulted in highly variable LPS levels [13]. In contrast to the CORDIOPREV study, in which the meals were the same for all participants and the fat content was adjusted by body weight [18], the participants of FINDRISC97 were only asked to avoid heavy meals. Thus, the meal was not standardized between participants in terms of fat or carbohydrate content, yet these parameters have been described to affect postprandial LPS levels [14].

In addition, in the CORDIOPREV study, the diagnosis of T2DM was performed according to the ADA diagnosis criteria [15], and assessed by clinicians at inclusion and every year, whereas the diagnosis in the FINDRISC97 cohort study was assessed by a self-administered questionnaire sent to the participants by mail, and physical measurements and blood sampling were carried out in primary care centers [37]. A more accurate T2DM diagnosis in the CORDIOPREV study, mainly in the diagnosis of the new cases of T2DM, may also be proven to increase the effectiveness of our dynamic test in the prediction of T2DM development.

Our results indicate that LPS postprandial changes improve the prediction of the risk of developing T2DM as compared with FINDRISC, a diabetes risk score based on anthropometric measures, blood pressure, physical activity and dietary habits. This should prove very useful to identify individuals at high risk for T2DM [23], and suggests that dynamic tests, such as a fat challenge, are more efficient than steady state-based measurements in assessing the phenotypic flexibility of an individual and the subsequent risk of developing metabolic disease. Notably, although individually HbA1c level was more efficient in T2DM risk prediction than the LPS postprandial increase, the LPS-FINDRISC combination was more efficient in T2DM risk prediction than the HbA1c-FINDRISC, and a high LPS postprandial fold change predictsT2DM development in patients with both high and low FINDRISC score. Moreover, our results are consistent with those obtained in animal models showing that subcutaneous infusion of LPS or high fat diet-induced endotoxemia triggers IR [38,39], and it also agrees with human studies pointing to IR as the initiating or primary defect in T2DM [4]. Our study also showed that the patients who developed T2DM after a median of follow-up of 60 months had higher postprandial endotoxemia together with lower insulin sensitivity at baseline, as shown by the insulin sensitivity indexes investigated. Taken together, our results suggest that an alteration in the intestinal barrier takes place before T2DM development, presumably by changes in gut microbiota, as evidenced by LBP plasma levels, which increased the LPS intestinal absorption, with the subsequent increases in TNF-a plasma levels. This is especially important, as TNF-a has been shown to cause IR by increasing serine phosphorylation on insulin receptor substrate-1 leading to its inactivation [40]. Thus, our results support the role of the intestine in the development of T2DM, as increased intestinal permeability may allow the absorption of pro-inflammatory bacterial components which induce inflammation and subsequently IR [10].

Our study has limitations. One limitation lies in the fact that the prevention of T2DM was not the primary endpoint of the CORDIOPREV trial, but was rather a secondary analysis conducted in the subgroup of cardiovascular patients without T2DM at baseline. In fact, the study included a large number of patients with acute myocardial infarction (AMI), which limits our findings to people with these characteristics and precludes its generalization to healthy individuals. Although diabetes prediction is very important, since patients with AMI and T2DM have a considerably higher risk of developing a new cardiovascular event than those without T2DM [41], validation in a cohort without cardiovascular disease and closer to general population would allow us to apply these methods to the general population.

Our results show that an elevated postprandial endotoxemia precedes the development of T2DM in patients with acute myocardial infarction and probably plays a role in promoting inflammation-induced insulin resistance and/or beta-cell dysfunction. In addition, our results also support the role of LPS plasma levels as a predictor biomarker and as a pathogenic factor in T2DM development. Further studies are needed to assess the potential changes in the integrity of the intestinal barrier that may precede T2DM development and the role of gut microbiota in this process.

Statement of authorship

A.C., R. J-L., J.F. A.-D., O.A. R.-Z., S. G.-C., J. L.-M. performed the experiments. A.C., R. J-L., J D-L, P.P-.M. drafted the manuscript. R B-R, J D-L, P.P-.M. performed the data analysis and results interpretation. J D-L, P.P-.M., B.v.O., J.M.O., M. M.-M., F.P.-J. and J.L.-M. contributed to the writing of the manuscript and revised it critically for important intellectual content. A.C., R. J-L., F.P.-J. and J.L.-M. conceived and designed the experiments. F.P.-J. and J.L.-M. have the responsibility for the contents of the article.

Conflict of interest

The author reports no conflicts of interest in this work.

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Granada, Spain, which performed the randomization process for this study.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.clnu.2018.03.016.

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2. Circulating miRNAs as Predictive Biomarkers of Type 2 Diabetes Mellitus Development in Coronary Heart Disease Patients:

from the CORDIOPREV Study

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Circulating miRNAs as Predictive Biomarkers of Type 2 Diabetes Mellitus Development in Coronary Heart Disease Patients from the CORDIOPREV Study

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Circulating microRNAs (miRNAs) have been proposed as type 2 diabetes biomarkers, and they may be a more sensitive way to predict development of the disease than the currently used tools. Our aim was to identify whether circulating miRNAs, added to clinical and biochemical markers, yielded better potential for predicting type 2 diabetes. The study included 462 non-diabetic patients at baseline in the CORDIOPREV study. After a median follow-up of 60 months, 107 of them developed type 2 diabetes. Plasma levels of 24 miRNAs were measured at baseline by qRT-PCR, and other strong biomarkers to predict diabetes were determined. The ROC analysis identified 9 miRNAs, which, added to HbA1c, have a greater predictive value in early diagnosis of type 2 diabetes (AUC = 0.8342) than HbA1c alone (AUC = 0.6950). The miRNA and HbA1c-based model did not improve when the FINDRISC was included (AUC = 0.8293). Cox regression analyses showed that patients with low *miR-103*, *miR-28-3p*, *miR-29a*, and *miR-9* and high *miR-30a-5p* and *miR-150* circulating levels have a higher risk of disease (HR = 11.27; 95% CI = 2.61–48.65). Our results suggest that circulating miRNAs could potentially be used as a new tool for predicting the development of type 2 diabetes in clinical practice.

INTRODUCTION

During the last decade, the prevalence of type 2 diabetes mellitus (T2DM) has been continuously on the rise; it has been estimated that the current number of T2DM patients worldwide will double in the next two decades[1] and similar trends have been reported for obesity.[2] It is, therefore, critical that there be early identification of individuals at high risk for developing diabetes in order to devise prevention strategies aimed at controlling the factors related to the development of the disease.[3,4]

The traditional biomarkers used to identify T2DM and pre-DM patients include glucose-homeostasis-related parameters, lipid classes, and scores such as the Finnish Diabetes Risk Score (FINDRISC).[5] In 2010, the American Diabetes Association (ADA) added glycated hemoglobin (HbA1c) as a diagnostic criterion for diabetes and prediabetes,[6,7] due to the strong association with the high risk of developing the disease. The use of HbA1c as a diagnostic test certainly has its advantages: convenience, less day-to-day variability, greater pre-analytical stability, and international standardization. Moreover, the FINDRISC has been successfully implemented as a practical screening test to assess the risk of diabetes and detect undiagnosed T2DM in European populations.[8,9] However, these parameters and tests also have their limitations and cannot precisely predict an individual's risk of developing T2DM, mainly because certain medical conditions may affect HbA1c and cause falsely high or low readings.[10,11] In addition, previous studies have examined the performance of HbA1c in comparison with fasting plasma glucose (FPG) in diagnosis of dysglycemia in older adults, and the authors have demonstrated considerable discrepancy between FPG- and HbA1c-based diagnoses of T2DM and pre-T2DM, with differences accentuated by race and gender.[12,13] It has also been demonstrated that the variability of HbA1c could be influenced by biological determinants of hemoglobin glycation, including epidemiological, genetic, and physiological factors.[12]

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It is, therefore, necessary to research into a new model that can use both HbA1c and the new biomarkers to improve sensitivity and specificity and increase the predictive value.

MicroRNAs (miRNAs) are autocrine and endocrine regulators of gene expression. In the latter case, it has been demonstrated that extracellular vesicles circulating in human plasma, which are a mixture of microparticles, exosomes, and other structures such as apoptotic bodies, contain miRNAs that can be considered as a novel class of signaling molecules mediating intercellular communication.[14] Some of these circulating miRNAs have been linked to glucose metabolism,[15] while others, such as *miR-144*, *miR-146a*, *miR-150*, *miR-182*, *miR-192*, *mir-29a*, *miR-30d*, and *miR-320*, seem to participate in the regulation of insulin signaling.[16,17] There are others, too, such as *miR-375*,[18–21] *miR-96*,[22] and *miR-124a*,[23] which have been associated with the development of T2DM and may also be involved in its progression.[24] One previous study has shown that a set of miRNAs, including *miR-15a*, *miR-29b*, *miR-126*, *miR-223*, and *miR-28-3p*, deregulated prior to the development of T2DM.[25] However, this study lacks a reliable predictive value, since it was performed in a reduced number of incident cases (n = 19), the T2DM diagnosis was not performed according to all the ADA diagnosis criteria,[26] and a poor statistical analysis was conducted.[25] Moreover, the idea about a circulating miRNA profile modulable by glucose homeostasis conditions is supported by two other studies, which related miRNA plasma levels with the grade of insulin resistance.[27,28]

Overall, there is evidence to suggest that a specific profile of circulating miRNAs could become a valuable biomarker to identify those normoglycemic and prediabetic individuals at increased risk of developing T2DM. However, until now, no studies have been carried out in a large population at high risk of developing T2DM to test this hypothesis.

Based on this previous evidence, our aim was to identify whether circulating miRNAs, added to clinical and biochemical markers (HbA1c, glucose, insulin, glucose after 2 hr of oral glucose tolerance test [OGTT], hepatic insulin resistance index [HIRI], insulinogenic index [IGI], insulin sensitivity index [ISI], disposition index [DI], muscle insulin sensitivity index [MISI], homeostatic model assessment-insulin resistance [HOMA-IR], and FINDRISC), would have the potential to predict new incident cases of T2DM more accurately.

RESULTS

Baseline Characteristics of the Patients

In the present work, from the full Coronary Diet Intervention with Olive Oil and Cardiovascular Prevention (CORDIOPREV) study population (1,002 patients), we included the 462 patients who at baseline had not been clinically diagnosed with T2DM.

At baseline, we observed higher values of waist circumference, body mass index (BMI), triglycerides (TGs), glucose, insulin, and HbA1c in the subgroup of patients who developed T2DM in a median follow-up of 60 months (Incident-T2DM group), compared with patients who did not develop T2DM (non-T2DM group) (all, $p < 0.05$). Moreover, our results showed lower baseline ISI, IGI, and DI and higher baseline HIRI and HOMA-IR values in the Incident-T2DM subgroup than in the non-T2DM group (all, $p < 0.05$). Conversely, we did not observe any differences in the baseline MISI (Table 1).

Bibliographic Identification of T2DM-Related Circulating miRNAs

Our bibliographic search of miRNAs associated with insulin sensitivity, insulin secretion and growth, and proliferation of beta cell yielded 24 miRNAs. When we measured the levels of miRNAs at baseline of the study (day 0 before dietary intervention), 17 were detected in the plasma of at least 80% of the patients, 4 were detected in less than 80% of the samples, and 3 were not detected at all in the plasma samples. (Table S1).

Multivariate Analysis by PCA and OPLS-DA

Whereas principal-components analysis (PCA) did not yield statistically significant results, the orthogonal partial least-squares discriminant analysis (OPLS-DA) showed differences between Incident-T2DM and non-T2DM (Q2 = 0.239; $R^2y = 0.270$ and $R^2x = 0.575$). Moreover, the variables importance projection (VIP) analysis identified 9 miRNAs (*miR-9*, *miR-28-3p*, *miR-29a*, *miR-30a-5p*, *miR-103*, *miR-126*, *miR-150*, *miR-223*, and *miR-375*) with VIP scores > 1, which supports their relevance in differentiating between Incident-T2DM and non-T2DM patients (Figure 1).

Baseline Levels of Circulating miRNAs

We observed that the baseline plasma levels of 9 miRNAs (*miR-9*, *miR-15a*, *miR-28-3p*, *miR-29a*, *miR-103*, *miR-223*, *miR-126*, *miR-145*, and *miR-375*) were significantly lower in Incident-T2DM patients than in non-T2DM patients. In contrast, the baseline plasma levels of 2 miRNAs (*miR-30a-5p* and *miR-150*) were significantly higher in Incident-T2DM as compared with non-T2DM patients. No significant differences between groups were found in the plasma levels of *miR-7*, *miR-10*, *miR-182*, and *miR-320* (Table 1).

Baseline Circulating Levels of miRNAs According to the Year of T2DM Diagnosis

From the 462 subjects included in this study, a total of 107 subjects developed T2DM (Incident-T2DM group) during the follow-up period, of whom 43 were diagnosed after the first year of follow-up, 24 in the second year, 11 in the third year, 19 in the fourth year, and 10 in the fifth year. The remaining group of subjects (355 subjects) did not develop the disease (non-T2DM group). To evaluate the influence of the time before the T2DM diagnosis on the predictive value of each baseline miRNA to identify individuals at high risk of developing the disease, we analyzed the baseline circulating levels of miRNAs in each subgroup of patients according to the year in which the diagnosis of T2DM took place, as compared with patients who did not develop T2DM. Overall, we observed lower baseline circulating levels of *miR-103*, *miR-28-3p*, *miR-9*, and *miR-29a* in subjects diagnosed with T2DM in the first 3 years of follow-up and higher levels of *miR-30a-5p* and *miR-150* in subjects diagnosed with T2DM in

Table 1. Baseline Characteristics and Circulating miRNA Levels of Subjects Who Did Not Develop T2DM (Non-T2DM) versus Subjects Who Developed T2DM (Incident-T2DM) after the Follow-up Period

Variable	Non-T2DM Group	Incident-T2DM Group	p Value	miRNAs	Non-T2DM Group	Incident-T2DM Group	p Value	q- Value
n	355	107	–	–	–	–	–	–
Age (years)	57 ± 0.5	59 ± 0.9	0.171	*miR-103*	103.9 ± 4.5	62.1 ± 4.8	<0.001	<0.001*
Waist circumference (cm)	101.7 ± 0.6	105.3 ± 1.1	0.003*	*miR-107*	1.70 ± 0.09	1.65 ± 0.17	0.810	0.853
BMI (kg/m^2)	29.9 ± 0.2	31.4 ± 0.5	0.002*	*miR-223*	79.0 ± 3.5	56.7 ± 3.6	0.016	0.032*
TG (mg/dL)	119.5 ± 3.2	132.6 ± 6.6	0.041*	*miR-29a*	68.1 ± 2.1	54.8 ± 4.0	<0.001	<0.001*
Total cholesterol (mg/dl)	160.6 ± 1.6	164.9 ± 3.4	0.288	*miR-28-3p*	185.9 ± 9.6	101.1 ± 8.8	<0.001	<0.001*
c-LDL (mg/dL)	91.1 ± 1.3	93.4 ± 2.7	0.473	*miR-126*	31.4 ± 1.6	18.8 ± 1.4	<0.001	<0.001*
c-HDL (mg/dL)	44.6 ± 0.5	43.5 ± 1.0	0.290	*miR-145*	5.10 ± 0.27	4.11 ± 0.43	0.013	0.021*
Apo A1 (mg/dL)	133.1 ± 1.2	135.2 ± 2.3	0.283	*miR-150*	0.69 ± 0.04	1.07 ± 0.09	<0.001	<0.001*
Apo B (mg/dL)	71.8 ± 1.0	76.3 ± 1.8	0.035*	*miR-15a*	2.70 ± 0.13	2.08 ± 0.28	<0.001	<0.001*
hs-CRP (mg/L)	2.51 ± 0.19	2.88 ± 0.29	0.031*	*miR-182*	58.7 ± 2.7	59.1 ± 4.9	0.7670	0.7887
Glucose (mg/dL)	92.6 ± 0.5	96.2 ± 1.1	0.002*	*miR-30a-5p*	25.6 ± 1.3	35.2 ± 2.5	<0.001	<0.001*
HbA1c (%)	5.8 ± 0.02	6.0 ± 0.03	<0.001*	*miR-320*	23.2 ± 0.9	24.0 ± 1.4	0.434	0.419
Insulin (mU/L)	8.33 ± 0.31	10.51 ± 0.66	0.007*	*miR-375*	20.9 ± 0.9	15.4 ± 1.1	0.013	0.020*
HIRI	1052 ± 37	1313 ± 90	0.002*	*miR-7*	6.38 ± 0.33	6.88 ± 0.58	0.398	0.425
MISI ($\times 10^2$)	2.10 ± 0.11	1.89 ± 0.16	0.369	*miR-9*	2.03 ± 0.15	0.93 ± 0.18	<0.001	0.002*
DI	1.01 ± 0.03	0.77 ± 0.04	<0.001*	–	–	–	–	–
ISI	4.30 ± 0.14	3.27 ± 0.18	<0.001*	–	–	–	–	–
IGI	1.14 ± 0.06	0.88 ± 0.07	0.025*	–	–	–	–	–
HOMA-IR	2.58 ± 0.09	3.23 ± 0.22	0.002*	–	–	–	–	–

Values expressed as mean ± SE. BMI, body mass index; c-HDL, high-density lipoprotein; c-LDL, low-density lipoprotein; TG, triglycerides; Apo A1, Apolipoprotein A1; Apo B, Apolipoprotein B; hs-CRP, high-sensitivity C-reactive protein; HbA1c, glycosylated hemoglobin; HIRI, hepatic insulin resistance index; MISI, muscle insulin sensitivity index; ISI, insulin sensitivity index; IGI, insulinogenic index; DI, disposition index; HOMA-IR, homeostasis model assessment-insulin resistance. Variables were calculated using one-way ANOVA through SPSS (now PASW Statistic for Windows, version 21.0) (IBM, Chicago, IL, USA). For data that did not fit the normal distribution, the Mann-Whitney U test was performed. *p < 0.05 in one-way ANOVA or after the false discovery rate (FDR) correction.

the first year of follow-up, compared with subjects diagnosed in later years and with the non-T2DM group (Figure 2).

Relationship between miRNA Plasma Levels and Insulin Sensitivity/Resistance and Beta Cell Function Indexes

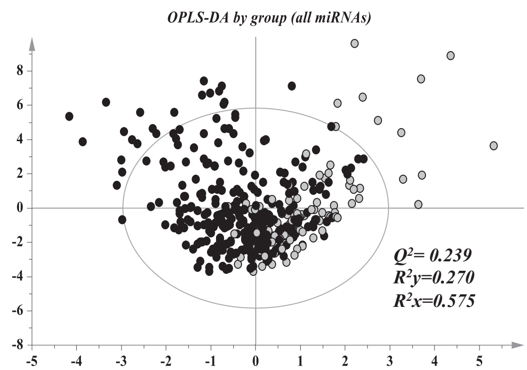
We studied the relationship between the plasma levels of the 9 miRNAs, identified by OPLS-DA, and the insulin sensitivity, beta cell function, and the insulin resistance of peripheral tissues, such as liver and muscle, by lineal regression methods, adjusted by age, gender, BMI, TGs, and high-density lipoprotein cholesterol (c-HDL). We observed that the ISI, DI, and IGI were related with the plasma levels of *miR-126*, *miR-103*, and *miR-150*, respectively. Moreover, we also observed a relationship between miRNAs and fasting glucose (*miR-28-3p*, *miR-30a-5p*, *miR-103*, *miR-126*, and *miR-150*) and HbA1c (*miR-28-3p*, *miR-103*, *miR-223*, and *miR-375*) (Table 2).

Received Operating Characteristic Curve Analysis

To evaluate the potential of miRNA plasma levels as a predictive biomarker of T2DM, the accuracy, sensitivity, and specificity were compared in 10 different models of received operating characteristic (ROC) analysis. We built models including all the miRNAs, measured together with the classic parameters (fasting glucose, 2-hr glucose, and HbA1c), beta cell function and insulin sensitivity/resistance indexes (IGI, ISI, DI, HIRI, and MISI), and the FINDRISC[5] (Table S2).

First, we included the full miRNA dataset (15 miRNAs) as input variables in the model, and we obtained an area under the curve (AUC) = 0.849 (95% confidence interval [CI] = 0.808–0.889). Further, we reduced the number of miRNAs included in the model according to the VIP score in the OPLS-DA. Thus, a model built including the 9 miRNAs with a VIP value of over 1 (*miR-9*, *miR-28-3p*, *miR-29a*, *miR-30a-5p*, *miR-103*, *miR-126*, *miR-150*, *miR-223*, and *miR-375*) yielded an only slightly reduced significance in the model AUC = 0.818 (95% CI = 0.771–0.864), as compared with the full 15 miRNA dataset.

Interestingly, the classic parameters, such as fasting glucose, insulin, 2-hr glucose (OGTT), and HbA1c, together with the insulin sensitivity indexes ISI, DI, IGI, HIRI, MIRI, HOMA-IR, and HOMA-beta yielded an AUC of 0.769 (95% CI = 0.713–0.826). In addition, the FINDRISC showed an AUC of 0.610 (95% CI = 0.550–0.671) (Figure 3A), which did not improve when we included insulin sensitivity indexes in the model AUC = 0.765 (95% CI = 0.709–0.820).



miRNAs	VIP score
hsa-miR-150	1.57283
hsa-miR-103	1.52393
hsa-miR-28-3p	1.48090
hsa-miR-126	1.33097
hsa-miR-9	1.14448
hsa-miR-30a-5p	1.09448
hsa-miR-223	1.04366
hsa-miR-375	1.04034
hsa-miR-29a	1.00829
hsa-miR-15a	0.62923
hsa-miR-145	0.29040
hsa-miR-7	0.23827
hsa-miR-320	0.05359
hsa-miR-182	0.04412
hsa-miR-107	0.03950

Figure 1. Orthogonal Partial Least-Squares Discriminant Analysis

Orthogonal partial least-squares discriminant analysis (OPLS-DA) was used to compare miRNA levels at baseline in order to analyze the differences between Incident-T2DM (gray circle) and non-T2DM patients (black circle) during follow-up. The quality of the models obtained by PCA and OPLS-DA was assessed by interrogation of the R^2 and Q2 parameters. The miRNAs with VIP score > 1 were considered important for differentiating between groups (table within the image). Data were processed using SIMCA-P+ (version 14.0.0.1359; Umetrics, Umea, Sweden).

To obtain an implemented miRNA-based model, we combined the dataset of 9 miRNAs with the HbA1c, which was the classic clinical parameter with greater weight in the Average Importance of Variables classification. Thus, the HbA1c showed an AUC of 0.676 (95% CI = 0.605–0.737), which improved after inclusion of the 9 miRNAs (AUC of 0.834; 95% CI = 0.790–0.878, sensitivity = 0.766, specificity = 0.809, and accuracy = 80.0%) (Figure 3A). In addition, the ROC analysis showed 3 miRNAs (*miR-9*, *miR-28-3p*, and *miR-29a*) with the highest differentiation power from the Average Importance of Variables classification, followed by HbA1c and *miR-150* (Figure 3B). Additionally, we built a model including the clinical parameters in the assessment of T2DM risk without miRNAs (fasting glucose, 2-hr glucose in OGTT, HbA1c, age, gender, BMI, c-HDL, TGs, diet, and waist circumference) showing an AUC = 0.7354 (Figure 3A).

Moreover, when we included the FINDRISC[5] together with the dataset of the 9 miRNAs and HbA1c, we did not improve the model (AUC of 0.829; 95% CI = 0.785–0.874, sensitivity = 0.796, specificity = 0.774, and accuracy = 77.9%), as compared with the one that included the dataset of 9 miRNAs and HbA1c.

Also, the AUCs of all models were compared using the DeLong test, and we observed a significant difference between the model based on clinical parameters without miRNAs (fasting glucose, 2-hr glucose in OGTT, HbA1c, age, gender, BMI, c-HDL, TGs, diet, and waist circumference) and the model based on 9 miRNAs and HbA1c (adjusted by age, gender, BMI, c-HDL, TGs, diet, and waist circumference) ($p = 0.01002$).

Internal validation by bootstrap resampling of the original set (1,000 randomized samples) in the best model (9 miRNAs + HbA1c) showed a degree of over-optimism of 0.03, which represents the deviation from the mean SE in estimation of these 1,000 samples (bootstrapped AUC ROC = 0.796).

Cox Regression Analysis

To test the T2DM-predictive value of miRNAs by the Cox regression analysis, we selected those with the highest VIP values in the OPLS-DA multivariate analysis: 9 miRNAs (*miR-9*, *miR-28-3p*, *miR-29a*, *miR-30a-5p*, *miR-103*, *miR-126*, *miR-150*, *miR-223*, and *miR-375*; Figure 1).

To perform the Cox regression analysis, we categorized subjects in tertiles according to the plasma levels for each miRNA as follows: low baseline plasma levels (T1), medium baseline plasma levels (T2), and high baseline plasma levels (T3). We compared the hazard ratio (HR) between T1 and T3 for each studied miRNA. We observed that low baseline plasma levels (T1) in 4 miRNAs (*miR-9*, *miR-28-3p*, *miR-29a*, and *miR-103*) and high plasma levels (T3) in 2 of 9 miRNAs (*miR-30a-5p* and *miR-150*) were associated with a high risk of T2DM development ($HR_{T1\ versus\ T3} \geq 2.5$) (Table 3).

Accordingly, to study the accumulative predictive value of the six miRNAs together, we included these six miRNAs with $HR_{T1\ versus\ T3} \geq 2.5$ and performed a Cox regression multi-miRNA analysis (Figure 4).

Next, we categorized patients according to the levels of these six miRNAs as follows: C1 category was composed of patients with low levels in at least three of the four downregulated miRNAs (*miR-9*, *miR-28-3p*, *miR-29a*, and *miR-103*) and high levels in at least one of the 2 upregulated miRNAs (*miR-150* and *miR-30a-5p*) (n = 46); C3 category was composed of patients with high levels in at least three of the four downregulated miRNAs and low levels in at least one of the two upregulated miRNAs (n = 32); and, finally, C2 was made up of patients with an intermediate miRNA deregulation profile

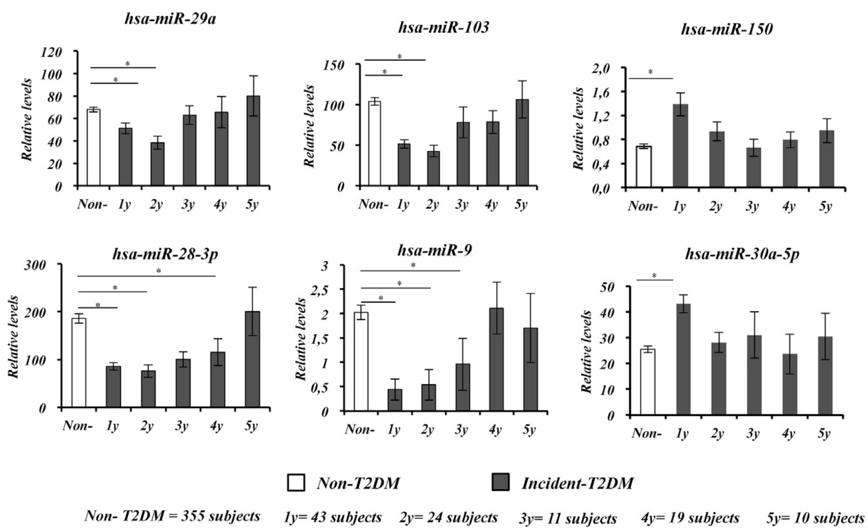


Figure 2. Circulating miRNA Levels at Baseline According to the Time Point at which Patients Were Diagnosed with T2DM

Data are represented as the mean ± SEM and correspond to the one-way ANOVA (in gray, subjects who developed T2DM [Incident-T2DM] and in white, subjects who did not develop T2DM [Non-T2DM]). The differences between groups were evaluated by Mann-Whitney U test. $^{*}p < 0.05$.

(n = 356). We therefore obtained an HR between C1 and C2 of 2.34 (95% CI = 0.74–7.42) and an HR between C1 and C3 of 11.68 (95% CI = 3.56–38.34). Likewise, when we adjusted the Cox regression analysis by age, gender, BMI, diet, HbA1c, waist circumference, TGs, c-HDL, IGI, HOMA-IR, and DI, we observed a HR for C1 versus C2 of 2.66 (95% CI = 0.64–11.07) and for C1 versus C3 of 11.27 (95% CI = 2.61–48.65) (Figure 5).

To compare our miRNA-based predictive model with the FINDRISC,[5] we categorized subjects into tertiles, according to the FINDRISC of our study as follows: low FINDRISC (T1), medium FINDRISC (T2), and high FINDRISC (T3). Next, we performed the Cox regression analysis and observed an HR between tertiles of FINDRISC T1 and T2 of 1.895 (95% CI = 1.131–3.174) and T1 and T3 of 2.362 (95% CI = 1.410–3.957) (Figure S1).

DISCUSSION

Our study showed that plasma levels of 9 of the 17 miRNAs selected with detectable levels at baseline of the study in most of our subjects were significantly associated with glucose metabolism and type 2 diabetes risk. These 9 miRNAs, in combination with HbA1c plasma levels, were able to differentiate between the patients who developed type 2 diabetes (Incident-T2DM) after a median follow-up of 60 months and those patients who did not develop the disease (Non-T2DM), with an AUC in the model of 0.834. The Cox regression analysis showed that patients with low *miR-103*, *miR-28-3p*, *miR-29a*, and *miR-9* and high *miR-30a-5p* and *miR-150* plasma levels were at higher risk of developing T2DM.

The relevance of the T2DM prevalence figures lies in the fact that subjects with T2DM have higher cardiovascular morbidity and mortality compared with non-diabetic subjects.[29] In addition, patients with acute myocardial infarction (AMI) and T2DM have a considerably higher risk of developing a new cardiovascular event than those without T2DM.[30] It is, therefore, very important to identify early on individuals with a higher risk of developing T2DM, in order to prevent the onset of the disease by using powerful therapies and efficiently controlling the associated cardiovascular risk factors.

Nowadays, the standard tools for identifying patients with a risk of T2DM include HbA1c and scores such as the FINDRISC. However, these tools are not able to effectively predict the disease development. Previous studies have revealed a number of limitations of HbA1c as a diagnostic test in comparison with FPG in the elderly, in which the lower sensitivity and specificity of HbA1c was demonstrated.[12,13] Moreover, it has recently become evident that FINDRISC is not universally applicable among all ethnic groups and populations.[31,32]

In addition, current evidence indicates that the blood miRNA profile may change under different pathophysiological conditions, such as cancer,[33] cardiovascular diseases,[34] and type 2 diabetes.[35]

In fact, two previous studies have related miRNA plasma levels with the grade of insulin resistance. One of them by Shah et al. identified 18 circulating miRNAs associated with plasma insulin and HOMA-IR levels,[28] of which 4 were also identified as being deregulated in the study by Zampetaki et al., who identified 13 miRNAs associated with the incidence of T2DM.[25] In our study, 9 miRNAs were identified as being important in the prediction model for T2DM, but only one matched with those identified by Zampetaki et al.[25] and by Shah et al.[28], suggesting that the differences in the phenotype between populations seem to be important in the deregulation of the miRNA profile associated with a pathological condition, such as glucose homeostasis.

In terms of T2DM prediction, the study by Zampetaki et al.[25] investigated miRNA plasma levels before the diagnosis of T2DM, which identified 5 miRNAs (*miR-15a*, *miR-28-3p*, *miR-29b*, *miR-126*, and *miR-223*) associated with the incidence of T2DM. Nevertheless, the small number of incident cases (n = 19) reported in that study precluded the more extensive statistical predictive analysis that we have carried out in our study (ROC and Cox regression analyses). In contrast, our study included all 462 non-diabetic patients of the CORDIOPREV at baseline of the study, of whom 107 developed T2DM in a median follow-up of 60 months, and the statistical

Table 2. Relationship between Circulating Levels of miRNAs and T2DM-Related Parameters

		hsa-miR103	*hsa-miR28-3p*	*hsa-mir-126*	*hsa-miR150*	*hsa-miR30a-5p*	*hsa-miR9*	*hsa-miR375*	*hsa-miR223*	*hsa-miR29a*
HbA1c (%)	r^2	−0.112	−0.122	−0.017	0.070	0.056	−0.049	−0.125	−0.091	−0.060
	p	0.014*	0.008*	0.369	0.085	0.134	0.168	0.007*	0.037*	0.121
Glucosa (mg/dL)	r^2	−0.129	−0.112	−0.099	0.107	0.109	−0.082	0.000	−0.039	0.019
	p	0.006*	0.014*	0.026*	0.018*	0.016*	0.054	0.497	0.220	0.354
Insulina (mU/L)	r^2	0.011	−0.034	−0.041	0.027	0.031	−0.008	−0.003	0.030	0.006
	p	0.413	0.250	0.209	0.300	0.274	0.436	0.478	0.276	0.455
HOMA-IR	r^2	0.016	−0.026	−0.013	0.010	0.001	0.059	0.027	−0.006	−0.040
	p	0.380	0.306	0.396	0.424	0.490	0.123	0.301	0.452	0.217
MISI	r^2	−0.080	0.025	0.037	−0.012	0.046	−0.069	−0.015	0.048	−0.023
	p	0.059	0.313	0.236	0.409	0.182	0.089	0.385	0.172	0.323
IGI	r^2	0.053	−0.016	−0.015	0.102	−0.021	0.080	−0.031	−0.013	0.030
	p	0.148	0.377	0.382	0.022*	0.340	0.060	0.269	0.398	0.280
ISI	r^2	0.029	0.077	0.102	−0.041	0.000	−0.015	0.008	0.078	0.003
	p	0.283	0.065	0.023*	0.213	0.499	0.444	0.441	0.062	0.478
DI	r^2	0.096	0.076	0.070	0.047	−0.045	0.054	−0.012	0.048	0.062
	p	0.030*	0.068	0.083	0.177	0.189	0.148	0.409	0.174	0.113
HIRI	r^2	0.016	−0.033	−0.024	0.021	0.004	0.063	0.024	−0.010	−0.042
	p	0.376	0.228	0.316	0.340	0.467	0.328	0.319	0.422	0.208

HbA1c, glycosylated hemoglobin; GLU, glucose; HOMA-B, homeostasis model assessment-beta cell function; HOMA-IR, homeostasis model assessment-insulin resistance; MIRI, muscle insulin resistance index; IGI, insulinogenic index; ISI, insulin sensitivity index; DI, disposition index; HIRI, hepatic insulin resistance index. Relationship was evaluated through a linear regression analysis adjusted by age, gender, body mass index (BMI), triglycerides (TG), and high-density lipoproteins (c-HDL), using SPSS (now PASW Statistic for Windows, version 21.0) (IBM, Chicago, IL, USA). *$p < 0.05$ in the linear regression analysis.

modeling (ROC and Cox regression models) allowed us to predict the onset of T2DM. In addition, in our study we followed ADA diagnosis criteria,[26] whereas the study by Zampetaki et al.[25] followed the World Health Organization guidelines, which do not take into account plasma levels of HbA1c as diagnosis criteria for diabetes and, therefore, did not produce a very accurate diagnosis.

The ROC analysis showed that HbA1c levels together with the set of 9 miRNAs were able to differentiate between Incident-T2DM and non-T2DM patients (AUC 0.834), and, furthermore, the model was more accurate than when only HbA1c (AUC 0.676) was used. In fact, the plasma levels of these 9 circulating miRNAs differentiate better between groups (Incident-T2DM versus non-T2DM) than the FINDRISC, on the basis of an AUC of 0.818 (9 miRNA plasma levels as input data) versus 0.610 obtained with the FINDRISC alone. It is worth noting that adding the FINDRISC to the 9-miRNA models (model 1: 9 miRNAs target + FINDRISC and model 2: 9 miRNAs target + FINDRISC + HbA1c) did not improve the accuracy in discriminating between the two groups of patients (AUC = 0.812 and AUC = 0.829, respectively).

Our results are in line with a recently published pilot cross-sectional study in which 8 miRNAs were able to discriminate between healthy people (n = 27), prediabetic patients (n = 12), type 1 diabetes patients (n = 16), and type 2 diabetes patients (n = 31),[36] supporting the idea that miRNA plasma levels could be used as disease biomarkers. However, the study by Syehan et al.[36] was performed in T2DM patients without a follow-up period, and, therefore, incidence was not analyzed, which means this study has no predictive value. More relevantly, our prospective study has shown the predictive value of measuring plasma miRNAs as biomarkers before T2DM development in patients at risk. Moreover, we studied different predictive models in a much larger population, including miRNAs for the first time as biomarkers. When these were added to the classic parameters, such as fasting glucose, insulin, 2-hr glucose (OGTT), HbA1c, insulin sensitivity indexes, and the FINDRISC, it allowed us to assess the risk of developing T2DM over the following 5 years.

Overall, after the multi-miRNA Cox regression analysis, we showed that the plasma levels of six miRNAs (*miR-9*, *miR-28-3p*, *miR-29a*, *miR-103*, *miR-30a-5p*, and *miR-150*) could potentially be used as predictive biomarkers, which is supported by their involvement in mechanisms related with the development of T2DM.

Circulating miRNAs are considered as a novel class of signaling molecules mediating intercellular communication,[14] and the blood miRNA profile may change under different pathophysiological conditions, which allows for their potential use as biomarkers in certain diseases such as T2DM.[25,33,35] However, circulating and intracellular miRNA levels may not match because, after the intracellular expression, miRNAs may be released,[37] and, therefore, a reduction in release could lead to intracellular accumulation but low plasma levels.

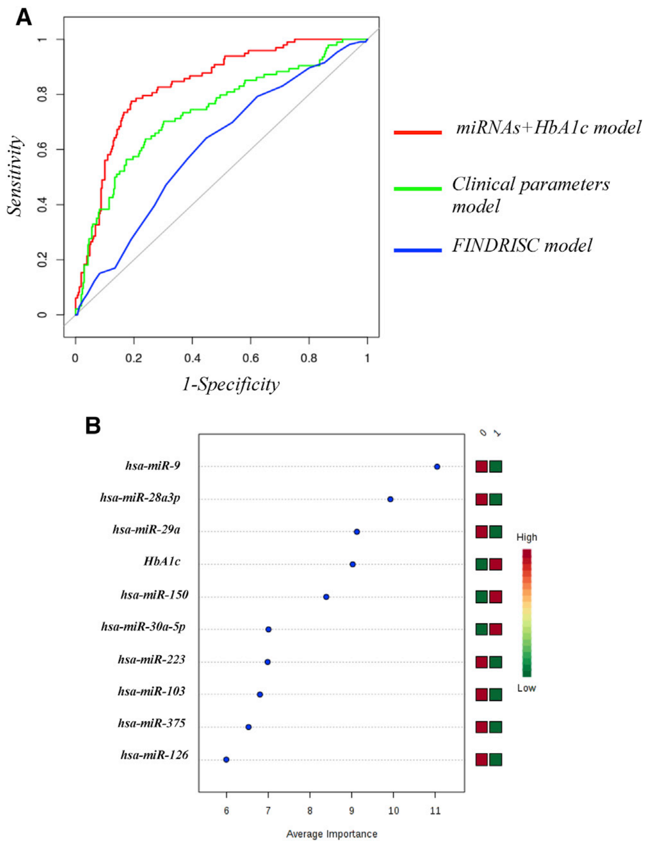


Figure 3. ROC Analysis and Classification by Average Importance of Variables Included in a ROC Model Based on miRNAs and HbA1c

(A) The comparison among the AUC of ROC curves of three models: red line, miRNA- and HbA1c-based model; green line, clinical parameters model; blue line, FINDRISC model. (B) The average importance classification of the variables included in the ROC curve can be seen. Non-T2DM = 0; Incident-T2DM = 1. Analysis was carried out by MetaboAnalyst 3.0.

Additionally, previous studies demonstrated the miRNA levels in the T2DM-diagnosed status, in contrast with our study in which we evaluated the profile of miRNAs years before disease development and diagnosis. In fact, previous evidence has shown that upregulation of *miR-29a* in INS-β cell mediates β cell dysfunction and could contribute to the progression from impaired glucose tolerance to type 2 diabetes.[38] Moreover, the inhibition of *miR-103* leads to improved glucose homeostasis and insulin sensitivity in obese mice[39] and an anti-inflammatory effect mediated by Cav1.[40] In addition, the overexpression of *miR-9* causes a decrease in glucose-stimulated insulin exocytosis by diminishing the expression of the transcription factor Onecut2, which represses the expression of granulophilin and negatively regulates insulin release.[41]

In the last few years, miRNA plasma levels have been shown to be useful as biomarkers for cardiovascular events[34] and cancer.[33] However, to the best of our knowledge, no studies have yet focused on the use of miRNA plasma levels as predictive biomarkers of T2DM development by combining a sample size large enough to obtain reliable results and robust statistical models. Thus, our study demonstrated for the first time that plasma levels of *miR-9*, *miR-28-3p*, *miR29a*, *miR-103*, *miR-30a-5p*, and *miR-150* are also powerful predictive biomarkers that can discriminate between Incident-T2DM and non-T2DM patients. More importantly, our results showed that the alteration in miRNA levels precedes the development of T2DM by 3 years, and, therefore, our model has a predictive power in this period of time.

Table 3. Hazard Ratio Observed after Cox Regression Analysis for Each miRNA Included in the ROC Curve

miRNA	HR T1 versus T1 (95% CI)	HR T1 versus T2 (95% CI)	HR T1 versus T3 (95% CI)
miR-150	1 (ref)	2.40 (1.30–4.43)	3.98[a] (2.21–7.17)
miR-103	1 (ref)	2.07 (1.15–3.71)	3.20[a] (1.85–5.55)
miR-28-3p	1 (ref)	2.83 (1.49–5.30)	4.45[a] (2.40–8.24)
miR-126	1 (ref)	1.82 (1.05–3.16)	2.28 (1.34–3.90)
miR-9	1 (ref)	0.99 (0.51–1.92)	3.95[a] (2.34–6.65)
miR-30a-5p	1 (ref)	1.37 (0.79–2.40)	2.53[a] (1.53–4.18)
miR-223	1 (ref)	2.56 (1.47–4.47)	1.90 (1.07–3.35)
miR-375	1 (ref)	1.78 (1.05–3.00)	1.54 (0.90–2.63)
miR-29a	1 (ref)	1.73 (0.97–3.07)	2.51[a] (1.46–4.33)

[a]miRNAs selected for multiple COX regression analyses.

Nonetheless, our study has limitations. One limitation lies in the fact that we carried out a focused bibliographic search of the miRNAs associated with insulin sensitivity, insulin secretion and growth, and proliferation of beta cells, which meant that any potential miRNAs whose implication with T2DM has not yet been described were not included in our model. In addition, T2DM prevention was not the primary endpoint of the CORDIOPREV trial, but it was a secondary analysis conducted in the subgroup of cardiovascular patients without T2DM at baseline. Finally, the study included a large number of elderly patients with AMI, which limits our findings to people with these characteristics and precludes generalization to healthy individuals. In line with this, futures studies should focus the validation of this study on another population. Although diabetes prediction is very important as patients with AMI and T2DM have a considerably higher risk of developing a new cardiovascular event than those without T2DM,[30] the validation in a cohort without cardiovascular disease and closer to the general population would allow us to adapt this method to the general population.

In conclusion, our results suggest that miRNA plasma levels added to HbA1c could become a valuable new tool for assessing the early risk of type 2 diabetes in clinical practice to prevent disease development.

MATERIALS AND METHODS

Study Subjects

This work was conducted within the framework of the CORDIOPREV study. The rationale, methods, and baseline characteristics have been reported by Delgado-Lista et al.,[42] and they are also provided in

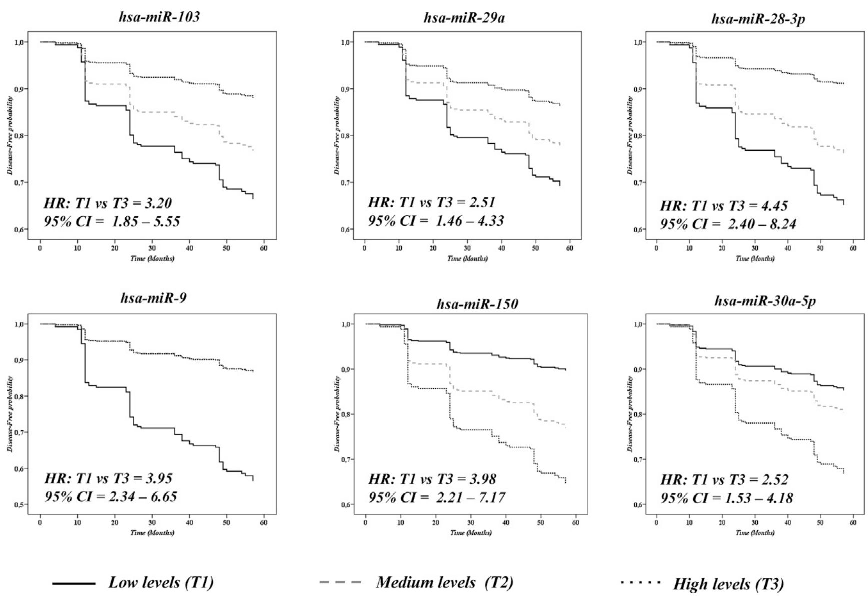


Figure 4. Disease-free Probability Analysis through a Cox Regression Model with the Six-miRNA Target

Data represent circulating levels for each miRNA by tertiles, low levels (T1), medium levels (T2), and high levels (T3). The analysis was carried out using SPPS (now PASW Statistic for Windows, version 21.0) (IBM, Chicago, IL, USA) and adjusted by diet, age, gender, BMI, TGs, c-HDL, HbA1c, and waist circumference.

ClinicalTrials.gov (NTC00924937). Briefly, the CORDIOPREV study is an ongoing prospective, randomized, single blind, controlled dietary intervention trial in 1,002 patients with coronary heart disease (CHD), high cardiovascular risk, aged between 20 and 75 years old, who had their last coronary event more than 6 months prior to enrollment and had no severe diseases or a life expectancy of less than 5 years. In addition to conventional treatment for CHD, the subjects were randomized into two different dietary models (Mediterranean and low-fat diets). The intervention phase is still in progress and will have a median follow-up of 7 years. The patients were recruited from November 2009 to February 2012, mostly at the Reina Sofia University Hospital (Córdoba, Spain), but patients from other hospital centers from the Córdoba and Jaen provinces were also admitted. Written consent was obtained from all the subjects prior to recruitment, and the study protocol and all amendments were approved by the Ethics Committee of Hospital Reina Sofia, all of which follow the Helsinki Declaration and good clinical practices.

In the present work, all the subjects (n = 462) who had not been clinically diagnosed with T2DM at baseline in the CORDIOPREV-DIAB study were included.[43] Of this group, 43 subjects were diagnosed as having T2DM after the first year of follow-up, 24 in the second year, 11 in the third year, 19 in the fourth year, and 10 in the fifth year, for a total of 107 subjects who developed T2DM (Incident-T2DM), according to all the ADA diagnosis criteria,[26] evaluated on the basis of glucose tolerance tests performed each year during the median follow-up of 60 months (Figure S2). Of the 462 subjects included in the current work, 216 were randomized to consume a low-fat high-complex carbohydrate diet (LFHCC diet) and 246 to consume a Mediterranean diet (Med diet). The differences between diets were evaluated by Chi-square test, and we observed no statistical significance (Chi2 = 1.948; p = 0.163). The baseline characteristics of the subjects in the study are shown in Table 1.

Biochemical Measurements of Metabolic Parameters

Venous blood from the participants was collected in tubes containing EDTA after a 12-hr overnight fast. Lipid variables were assessed with the DDPPII Hitachi modular auto analyzer (Roche, Basel, Switzerland) using specific reagents (Boehringer-Mannheim, Mannheim, Germany). Measurements of total cholesterol (TC) and TG levels were performed by colorimetric enzymatic methods,[44,45] c-HDL was measured by colorimetric assay,[46] and low-density lipoprotein (c-LDL) concentration was calculated by the Friedewald equation, using the following formula: c-LDL = CT − (c-HDL + TG/5). Glucose measurements were performed using the hexokinase method. The hs-C-Reactive Protein (hs-CRP) was determined by high-sensitivity ELISA (BioCheck, Foster City, CA, USA). Plasma insulin concentrations were measured by microparticle enzyme immunoassay (Abbott Diagnostics, Matsudo-shi, Japan). Non-esterified fatty acid concentrations were measured by enzymatic colorimetric assay (Roche Diagnostics, Penzberg, Germany). ApoA-1 and ApoB concentrations were determined by immunoturbidimetry.

Estimation of IR, Insulin Secretion, and Beta Cell Function Indexes and FINDRISC

Before starting the test, the patients had fasted (from food and drugs) for 12 hr, and they were asked to refrain from smoking during the fasting period and from alcohol intake during the preceding 7 days. They were also asked to avoid strenuous physical activity the day before the test was given. At 8:00 a.m., the patients were admitted into the laboratory to perform the OGTT (75 g dextrose monohydrate in 250 mL water, NUTER. TEC GLUCOSA 50), and 0-, 30-, 60-, and 120-min sampling was performed to establish plasma glucose and insulin levels.

The Matsuda ISI was calculated from the OGTT using the following formula: ISI = 10.000 ÷ $\sqrt{}$([fasting plasma glucose × fasting plasma insulin] × [mean glucose in OGTT × mean insulin in OGTT]).[47] HOMA-IR was calculated as previously described by Song et al.[48] Insulin secretion was measured by the IGI as follows: IGI = [30 min insulin − fasting insulin (pmol/L)]/[30 min glucose − fasting glucose (mmol/L)].[49] Beta cell function was estimated by calculating the DI as follows: DI = ISI × [AUC30 min insulin/AUC30 min glucose], where AUC30 min is the AUC between baseline and 30 min of the OGTT for insulin (pmol/L) and glucose (mmol/L) measurements,

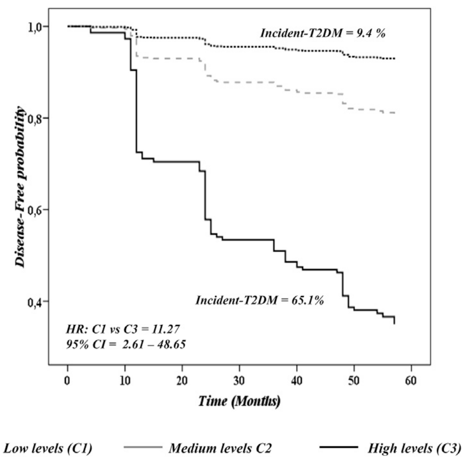


Figure 5. Disease-free Analysis through a Cox Regression Model Based on Multi-miRNAs, Including *miR-103*, *miR-28-3p*, *miR-29a*, *miR-9*, *miR-150*, and *miR-30a-5p*

The data represent circulating levels of all six miRNAs together; subjects were, therefore, classified into three categories as follows: C1 category was composed of patients with low levels in at least three of the four downregulated miRNAs (*miR-9*, *miR-28-3p*, *miR-29a*, and *miR-103*) and high levels in at least one of the 2 upregulated miRNAs (*miR-150* and *miR-30a-5p*) (n = 46); C3 category was composed of patients with high levels in at least three of the four downregulated miRNAs and low levels in at least one of the two upregulated miRNAs (n = 32); and, finally, C2 was made up of patients with an intermediate miRNA deregulation profile (n = 356). The analysis was carried out through SPPS (now PASW Statistic for Windows, version 21.0) (IBM, Chicago, IL, USA) and adjusted by age, gender, BMI, diet, HbA1c, waist circumference, TGs, c-HDL, IGI, HOMA-IR, and DI.

respectively, calculated by the trapezoidal method.[50] The indices used to determine tissue-specific IR were the HIRI and the MISI, which were calculated as described in previous work by our group,[43] following the methods described by Matsuda and DeFronzo[47] for HIRI and Abdul-Ghani et al. for MISI.[51] The FINDRISC was calculated as defined by Lindström et al.[5]

Isolation of Circulating miRNAs from Plasma Samples

Total RNA was isolated from plasma using the miRNeasy Mini Kit (QIAGEN, Hilden, Germany). Briefly, venous blood from the participants was collected at baseline (day 0 before dietary intervention) in tubes containing EDTA and centrifuged at 2,000 × *g* for 10 min for plasma separation from blood cells. Further, 200 μL EDTA-plasma was mixed with 1 mL Qiazol, incubated for 5 min at room temperature, and subsequently mixed with 200 μL chloroform. We added 2 μg MS2 RNA carrier (Roche, Mannheim, Germany) before the chloroform protocol step. The organic and aqueous phases were separated by centrifugation at 12,000 × *g* for 15 min at 4°C. The aqueous phase was collected and the RNA was precipitated by adding 100% ethanol. The mixture was applied to an miRNeasy Mini spin column and was centrifuged at 8,000 × *g* for 2 min. Next, 700 μL RWT buffer was added to the RNeasy MinElute spin column at 8,000 × *g* for 2 min. It was then washed again with 500 μL RPE buffer and 500 μL 80% ethanol. RNA was eluted in 14 μL RNase-free water. RNA purity and concentration were evaluated by spectrophotometry using NanoDrop ND-2000 (Thermo Fisher Scientific, Waltham, MA, USA).

miRNA Retrotranscription and Preamplification

The miRNA expression study was carried out on 24 miRNAs, which, based on a bibliographic search, were selected according to their association with insulin sensitivity, insulin secretion, inflammation, and growth and proliferation of beta cells (Table S1). The retrotranscription of RNA was carried out using the TaqMan MicroRNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA). RT mix contained 2 μL RNA and 3 μL RT custom primer pool in a final volume of 7.5 μL. RT primer pool was customized selecting specific primers for our set of target miRNAs in the database (https://www.thermofisher.com/es/en/home/life-science/pcr/real-time-pcr/real-time-pcr-assays/mirna-ncrna-taqman-assays.html). Plates were incubated in the iQ5 thermocycler (Bio-Rad, Hercules, CA, USA) at 16°C for 30 min, followed by 42°C for 30 min, and finally at 85°C for 5 min. In this step, the cDNA was stored at −20°C for a maximum of 1 week. Then, we prepared a mixture containing 10 μL customized PreAmp primer pool specific for our set of target miRNAs and 7.5 μL RT mix and 20 μL TaqMan PreAmp Master Mix (Life Technologies, Carlsbad, CA, USA) to a final volume of 40 μL. Next, the mixture was incubated in the iQ5 Thermocycler using the following steps: denaturation at 95°C for 10 min, 55°C for 2 min, and 72°C for 2 min; 20 cycles of amplification for 15 s at 95°C and 4 min at 60°C per cycle; and finally incubation at 99.9°C for 10 min. The pre-amplified products were then diluted with RNase-free water at a ratio of 1:40 and used for the real-time RT-PCR reactions.

Levels of Circulating miRNAs by Real-Time PCR

We measured the levels of miRNAs at baseline of the study with the OpenArray platform (Life Technologies, Carlsbad, CA, USA), following the manufacturer's instructions. As a normalization method, we first selected the miRNAs that showed the least variability in their CT values in all samples. For this, we used the NormFinder Bioinformatic tool (MOMA-Department of Molecular Medicine, Aarhus University Hospital, Denmark),[52] a software extensively used in expression studies.[53,54] The application showed that the most stable miRNAs were *miR-143* and *miR-144*. Second, we used the BestKeeper method to calculate the geometric mean of the pairwise Ct values (Ct values of *miR-143* and *miR-144*).[55] The relative expression data were analyzed using OpenArray Real-Time qPCR Analysis Software (Life Technologies, Carlsbad, CA, USA).

PCA and OPLS-DA

Prior to the PCA, the data were normalized into a dataset suitable for analysis. Applying the procedures of mean-centering and unit variance (UV) scaling, the data of the miRNA levels were processed using SIMCA-P+ (version 14.0.0.1359; Umetrics, Umea, Sweden). PCA was applied to the dataset and the score plots were visually inspected for the detection of patterns and outliers. OPLS-DA was used to compare miRNA levels at baseline, in order to analyze the differences between Incident-T2DM and non-T2DM patients during follow-up. OPLS-DA validation was performed by cross-validation (CV) method in the SIMCA-P+ software using the default setting, which includes a procedure of 7-fold cross-validation where the dataset is split into 7 different subsets.[56]The quality of the models obtained by PCA and OPLS-DA was assessed by interrogation of the R^2 and Q^2 parameters.[56,57] Next, we selected those miRNAs with higher discriminatory power between groups from the VIP score obtained in the OPLS-DA model. The miRNAs with a VIP score > 1 were considered important for differentiating between groups.

Statistical Analysis

The quantitative data were evaluated for normal distribution by the Kolmogorov-Smirnov test, with the cutoff for normal distribution set at $p > 0.05$. For data that were not normally distributed, we used the Mann-Whitney U test. The relationship between miRNA plasma levels and insulin sensitivity/resistance and beta cell function indexes was evaluated through a linear regression analysis adjusted by age, gender, BMI, TGs, and c-HDL. We used the Cox proportional hazard regression analysis to test the potential predictive value of the miRNAs studied. The level values of each miRNA were categorized by tertiles: low levels (T1), medium levels (T2), and high levels (T3). The *HR* in the analysis of each miRNA studied was analyzed by comparing T1 versus T2 and T1 versus T3. Six miRNAs with $HR_{T1\ versus\ T3} \geq 2.5$ were selected for Cox regression multi-miRNA analysis. The subjects were, therefore, classified into three categories: C1 category was composed of patients with low levels in at least three of the four downregulated miRNAs (*miR-9*, *miR-28-3p*, *miR-29a*, and *miR-103*) and high levels in at least one of the 2 upregulated miRNAs (*miR-150* and *miR-30a-5p*) (n = 46); C3 category was composed of patients with high levels in at least three of the four downregulated miRNAs and low levels in at least one of the two upregulated miRNAs (n = 32); and, finally, C2 was made up of patients with an intermediate miRNA deregulation profile (n = 356). This classification was called *6miRNAs-variable* and was included in the Cox regression multi-miRNA analysis. The HR in the analysis between C1 versus C2 and C1 versus C3 was compared. The lineal regression and Cox regression analyses were adjusted by age, gender, diet, HbA1c, BMI, TGs, c-HDL, and waist circumference. p values ≤ 0.05 were considered statistically significant. We used ROC analysis to estimate the AUC, accuracy, specificity, and sensitivity of the variables for differentiation of Incident-T2DM from non-T2DM patients. The models were corrected for those covariables that were allowed, avoiding overestimating information, the set of covariables included: diet, age, gender, BMI, c-HDL, TGs, HbA1c, and waist circumference. For internal validation of the model, the degree of over-optimism was estimated using bootstrap resampling of the original set (1,000 randomized samples).

All the statistical analyses were carried out using SPSS (now PASW Statistic for Windows, version 21.0) (IBM, Chicago, IL, USA). Additionally, we used MetaboAnalyst 3.0 to classify the variables included in the ROC model according to the average importance of variables. Data normalization was performed using the auto-scaling method, based on mean centered and divided by the SD of each variable.[58,59] The DeLong test was performed to compare the ROC models as described in DeLong et al.[60] for paired ROC curves using the software R.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and two tables and can be found with this article online at https://doi.org/10.1016/j.omtn.2018.05.002.

AUTHOR CONTRIBUTIONS

R.J.-L., O.A.R.-Z., A.C., and J. López-Miranda. conceived and designed the experiments. J.F.A.-D., J.D.-L., P.P.-M., J. López-Moreno, and J. López-Miranda participated in the recruitment and carried out the clinical and nutritional control of the volunteers. J.C.-V. was responsible for the management of samples and laboratory biochemical determinations. R.J.-L., A.C., and O.A.R.-Z. performed the experiments and collected the data. R.J.-L., O.A.R.-Z., J.F.A.-D., I.R.-R., E.M.Y.-S., A.C., and J. López-Miranda analyzed and interpreted the data. R.J.-L., O.A.R.-Z., A.C., and J. López-Miranda drafted the manuscript. J. López-Miranda conceived and designed the study. H.M.-A. supported the statistical analysis of data. J.P.C., J.M.O., and J. López-Miranda provided critical revision of the paper for the important intellectual content. J.D.-L., P.P.-M., A.C., and J. López-Miranda had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. All the authors were involved in writing the paper and gave their final approval to the submitted and published versions.

CONFLICTS OF INTEREST

The authors have no conflict of interest.

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3. A plasma circulating miRNAs profile predicts Type 2 Diabetes Mellitus and prediabetes: from the CORDIOPREV study

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20th Sep 18
Dear Prof López-Miranda,

Thank you for your submission of manuscript EMM2017768RRR entitled A plasma circulating miRNAs profile predicts type 2 diabetes mellitus and prediabetes: from the CORDIOPREV study to Experimental & Molecular Medicine. I am pleased to inform you that your manuscript has been accepted in its current form for publication in Experimental & Molecular Medicine. Please find the reviewer's comments on this manuscript at the end of this letter. Your paper is considered to be a significant contribution to the field, and we appreciate the opportunity to publish it in this journal.

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Comments from the Reviewer #1 (Comments to the Author):

The authors have addressed our concerns, especially with regards to mechanism. This is now an interesting association paper. They make a good point about the relative predictive value of OGTT vs. mirRNA profile, and its application in the clinic.

A plasma circulating miRNAs profile predicts type 2 diabetes mellitus and prediabetes: from the CORDIOPREV study.

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Running Title: Plasma miRNAs as T2DM and Pre-DM biomarkers

Clinical Trial Registration number: NCT00924937.

ABSTRACT

We aimed to explore whether changes in circulating levels of miRNAs according to type 2 diabetes mellitus or prediabetes status could be used as biomarkers to evaluate the risk of developing the disease. The study included 462 patients without disease at baseline from the CORDIOPREV trial. After a median follow-up of 60 months, 107 of the subjects developed the disease, 30 prediabetes, 223 maintained prediabetes and 78 remained disease-free. Plasma levels of four miRNAs related with insulin signaling and beta-cell function were measured by RT-PCR. We analyzed the relationship between miRNAs levels and insulin signaling and release indexes at baseline and after the follow-up period. The risk of developing disease based on tertiles (T1-T2-T3) of baseline miRNAs levels was evaluated by COX analysis. Thus, we observed higher *miR-150* and *miR-30a-5p* and lower *miR-15a* and *miR-375* baseline levels in type 2 diabetes than disease-free subjects. Patients with high *miR-150* and *miR-30a-5p* baseline levels had lower disposition index (p=0.047 and p= 0.007, respectively). The higher risk of disease was associated with high levels (T3) of *miR-150* and *miR-30a-5p* ($HR_{T3\text{-}T1}$ = 4.218 and $HR_{T3\text{-}T1}$ = 2.527, respectively), and low levels (T1) of *miR-15a* and *miR-375* ($HR_{T1\text{-}T3}$ = 3.269 and $HR_{T1\text{-}T3}$ = 1.604, respectively). In conclusion, our study showed that deregulated plasma levels of *miR-150*, *miR-30a-5p*, *miR-15a*, and *miR-375* were observed years before the onset of T2DM and pre-DM and could be used to evaluate the risk of developing the disease, which may improve prediction and prevention among individuals at high risk of T2DM.

Keywords: miRNAs; T2DM; prediabetes; biomarkers; disease biomarkers.

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a major public health problem and its prevalence has been increasing over the past few decades with no signs of receding in the near future. In addition to the personal affliction, this disease imposes a heavy economic burden on the global health-care system and overall economy [1].

In prediabetic conditions, beta cells compensate the insulin-resistant state from target tissues by increasing both the mass of pancreatic islets and the release of insulin in response to dietary glucose and fatty acids [2]. Chronic stress conditions may induce the activation of adaptation processes which exceed the normal phenotypic flexibility leading to progressive inflexibility [3] and to beta-cell dysfunction, followed by elevated fasting glucose (IFG) and/or impaired glucose tolerance (IGT) [4, 5]. Thus, the degree of hyperglycemia reflects the severity of the metabolic process, suggesting that it is an important marker to determine the developmental stage of the disease [6, 7].

However, T2DM frequently goes undiagnosed for many years because the hyperglycemia develops gradually and, in its earlier stages, it is not severe enough for the patient to note any of the classic symptoms of diabetes [8]. For this reason, the tools used currently to screen and diagnose T2DM and to detect individuals with prediabetes (Pre-DM) do not adequately predict the onset of the disease and monitor its progression [9]. Therefore, more informative biomarkers are needed to identify beta-cell injury and to assess the risk of disease, monitor responses to treatment, personalize therapy and improve the patients' quality of life [10].

Nowadays, miRNAs are recognized as important regulators of gene expression and central players in the control of several biological and pathological processes, including T2DM [11]. To this end, in vitro models have shown that *miR-30a-5p*[12] and *miR-144* play a role in beta-cell dysfunction [13]. Moreover, the plasma concentrations of individual [14] and multiple circulating miRNAs have been identified as being significantly different between T2DM patients and pre-diabetic subjects [15, 16]. In addition, alterations in plasma miRNAs levels have been observed in other metabolic diseases such as metabolic syndrome [15, 17]. However, those experimental designs were not able to identify whether the differences in miRNA levels found were the cause or a consequence of the development of metabolic disease. To the best of our knowledge, only two studies have observed alterations in plasma miRNAs levels before T2DM development [18, 19].

This calls for the need to conduct long-term follow-up studies in larger populations with incident cases of T2DM to identify the role of microRNA during the development of T2DM and to use that information to assist in the development of predictive biomarkers of T2DM. Therefore, in this study, we aimed to study whether plasma circulating levels of miRNAs according to T2DM or pre-DM status could be used as biomarkers to evaluate the risk of developing the disease.

MATERIALS AND METHODS

Study subjects

This work was conducted within the framework of the CORDIOPREV study, whose rationale, methods and baseline characteristics have been reported by Delgado-Lista *et al* [20]. Briefly, the CORDIOPREV study is an ongoing prospective, randomized, single-blind, controlled dietary intervention trial in 1002 coronary health disease (CHD) patients with a high risk of cardiovascular disease, who had their last coronary event more than six months before enrolment. In addition to conventional treatment for CHD, the subjects were randomized into two different dietary models (Mediterranean and low-fat diets). The intervention phase is still in progress and will have a median follow-up of seven years. Patients were recruited from November 2009 to February 2012, mostly at the Reina Sofia University Hospital (Córdoba, Spain), but patients from other hospitals from the Córdoba and Jaen provinces were also admitted. In summary, the patients were eligible if they were between 20 and 75, had established CHD without any clinical events in the last six months, were thought to follow a long-term dietary intervention and had no severe diseases or an expected life expectancy of five years. Details of the trial design were provided in Clinicaltrials.gov (NTC00924937). Written consent was obtained from all the subjects prior to the recruitment and the study protocol and all the amendments were approved by the Ethics Committee of Hospital Reina Sofia, all of which follow the Helsinki Declaration and good clinical practices.

All patients from the CORDIOPREV-DIAB without T2DM at baseline according to the ADA diagnostic criteria [21] (N=462) were included in this study [22]. T2DM and Pre-DM was diagnosed according to the American Diabetes Association (ADA) diagnosis criteria [21] and was evaluated by glucose tolerance tests performed each year during the five years of follow-up. The pre-diabetic status was defined as having one or more of the following criteria present in a participant: fasting plasma glucose (FPG) concentration ≥100 and < 126 mg/dL, impaired fasting glucose (IFG); FPG ≥140 and < 200 mg/dL 2h after an oral glucose test (OGTT), impaired glucose tolerance (IGT) [23] and glycosylated hemoglobin (HbA1c) ≥5.7 and <6.4 % [24].

In a median follow-up of 60 months, 78 subjects did not develop T2DM or pre-DM (non-T2DM); 239 subjects were pre-DM at baseline, of which 223 subjects maintained pre-DM status during the follow-up period (pre-DM); 30 subjects were not pre-DM at baseline but developed pre-DM in the follow-up period (incident pre-DM). Finally, 107 of the participating subjects developed T2DM (incident-T2DM) in a median follow-up of 60 months. Moreover 24 patients were not included in this study due to declines, died and withdrew for other reasons. The baseline characteristics of the subjects in the study are shown in **Table 1**.

Biochemical measurements of metabolic parameters

Venous blood from the participants was collected in tubes containing EDTA after a 12-h overnight fast. Lipid variables were assessed with the modular auto analyzer DDPPII Hitachi (Roche, Basel, Switzerland) using specific reagents (Boehringer-Mannheim, Mannheim, Germany). Measurements of total cholesterol (TC) and triglycerides (TG) levels were made by colorimetric enzymatic methods [25, 26]; high-density lipoprotein-cholesterol (HDL-c) by colorimetric assay [27]; and the low-density lipoprotein (LDL-C) concentration was calculated by the Friedewald equation, using the following formula: LDL-C = CT - (HDL-C + TG/5). Glucose measurements were performed using the hexokinase method. The hs-C-Reactive Protein (hs-CRP) was determined by high-sensitivity ELISA (BioCheck, Inc., Foster City, CA, USA). Plasma insulin concentrations were measured by microparticle enzyme immunoassay (Abbott

Diagnostics, Matsudo-shi, Japan). Non-esterified fatty acid concentrations were measured by enzymatic colorimetric assay (Roche Diagnostics, Penzberg, Germany). The ApoA-1 and ApoB concentrations were determined by immunoturbidimetry.

Insulin signaling and release indexes

Before starting the test, the patients abstained from food and medications for 12h and were asked to refrain from smoking during the fasting period and from alcohol intake during the preceding seven days. They were also requested to avoid strenuous physical activity the day before the test was administered. Oral glucose tolerance test (OGTT): At 8:00 a.m., the patients were admitted to the laboratory and an OGTT (75 g dextrose monohydrate in 250 ml water, NUTER. TEC GLUCOSA 50) was performed with 0, 30, 60, and 120 min sampling to establish plasma glucose and insulin levels [28].

The insulin sensitivity index (ISI) was calculated in the OGTT using the following formula: ISI = 10.000 ÷ √([fasting plasma glucose X fasting plasma insulin] X [mean glucose in OGTT X mean insulin in OGTT]) [28]. HOMA-IR was calculated as previously described by Song, Y., *et al.* [29]. Insulin secretion was measured using the insulinogenic index (IGI): IGI= [30 min insulin−fasting insulin (pmol/l)]/[30 min glucose−fasting glucose (mmol/l) [30]. The disposition index (DI) was estimated as follows: DI=ISI×[AUC30 min insulin / AUC30 min glucose], where AUC30 min is the area under the curve between the baseline and 30 min of the OGTT for insulin (pmol/l) and glucose (mmol/l) measurements, respectively, calculated by the trapezoidal method [31]. The hepatic insulin resistance index (HIRI) and the muscle insulin sensitivity index (MISI) were used to evaluate the tissue-specific IR as described in previous work by our group [22], following the methods described by Matsuda and DeFronzo for HIRI and Abdul-Ghani *et al.* for MISI [28, 32].

Isolation of circulating miRNAs

Circulating miRNAs were isolated from plasma samples, which were obtained using an EDTA-blood tube. In this way, venous blood from the participants was obtained by venipuncture and kept on ice until centrifugation. Immediately, in order to separate the plasma from the erythrocytes and the buffy coat fractions, EDTA-blood tubes were centrifugated at 2.000 x g for 10 min at 4ºC. The superior phase corresponding to the plasma was carefully separated using a pasteur pipette and the sample was stored at -80 ° C until use. The plasma samples were then defrosted on ice for RNA isolation.

The total RNA from the plasma was isolated using miRNeasy Mini Kit (Qiagen, Hilden, Germany). In short, 200 µL of EDTA-plasma was mixed with 1 mL of Qiazol, incubated for 5 min at room temperature and then mixed with 200 µL of chloroform. We added 2 µg of MS2 RNA carrier (Roche. Mannheim, Germany) before the chloroform protocol step. The organic and aqueous phase was separated by centrifugation at 12,000 *g* for 15 min, at 4ºC. The aqueous phase was collected, and the RNA was precipitated by adding 100% ethanol. The mixture was applied to a miRNeasy Mini spin column and was centrifuged at 8,000 *g* for 2 min. Next, 700 µL of RWT buffer was added to the RNeasy MinElute spin column at 8000 *g* for 2 min. We then washed it with 500 µL of RPE buffer and 500 µL of 80% ethanol. RNA was eluted in 14 µL RNase-free water. RNA purity and concentration were evaluated by spectrophotometry using NanoDrop ND-2000 (ThermoFisher, Waltham, MA).

Retrotranscription and preamplification of miRNAs

The study of miRNAs expression was carried out in four miRNAs (*miR-150; miR-30a-5p; miR-15a and miR-375)* selected on the basis of previous evidence of their participation on the biomechanisms involved in T2DM pathogenesis - in particular, beta cell function, insulin secretion and insulin sensitivity [12, 33-37]. The retrotranscription of RNA was carried out using the TaqMan MicroRNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA). The RT mix contained 2 µL of RNA and 3 µL of RT custom primer pool in a final volume of 7.5 µL. The RT primer pool was customized by selecting specific primers for our set of target miRNAs on the database (https://www.thermofisher.com/es/en/home/life-science/pcr/real-time-pcr/real-time-pcr-assays/mirna-ncrna-taqman-assays.html). The plates were incubated in the iQ5 thermocycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 16ºC for 30 min, followed by 42ºC for 30 min, and finally at 85ºC for 5 min. In this step, the cDNA could be stored at -20 ºC for one week. We then prepared a mixture containing 10 µl of PreAmp custom primers pool specific to our set of target miRNAs, 7.5 µL of RT mix and 20 µL of TaqMan PreAmp Master Mix (Life Technologies, Carlsbad, CA, USA) to make a final volume of 40 µL. The mixture was then incubated in the iQ5 Thermocycler after the following steps: denaturation at 95°C for 10 min; 55ºC for 2 min, 72ºC for 2 min; 20 cycles of amplification (15 s at 95°C, 4 min at 60°C) and finally 99.9ºC for 10 min. The pre-amplified products were then diluted with RNase-free water at a ratio of 1:40 and used for real-time RT-PCR reactions.

Measuring levels of circulating miRNAs using real-time PCR

The circulating levels of miRNAs were measured through the OpenArray® platform (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. As a normalization method, we first selected the miRNAs that showed the least variability in their CT values in all samples. For this, we used the NormFinder Bioinformatic tool (MOMA-Department of Molecular Medicine, Aarhus University Hospital, Denmark) [38](software extensively used in expression studies [39, 40]). The application showed that the most stable miRNAs were *miR-143* and *miR-144*. Secondly, we used the BestKeeper method to calculate the geometric mean of the pair-wise Ct values (Ct values of *miR-143* and *miR-144*)[41]. The relative expression data were analyzed by OpenArray® Real-Time qPCR Analysis Software and with the relative quantification powered by Thermo Fisher Cloud (Life Technologies, Carlsbad, CA, USA). MiRNAs levels are expressed in arbitrary units (AU), calculated with the CT of each target miRNAS with reference to the BestKeeper value.

Relationship of insulin signaling and release indexes with circulating miRNA levels

To analyze the relationship between baseline plasma miRNA levels and both insulin signaling and release indexes, the subjects were categorized by the median of their plasma miRNA levels. Thus, for each miRNA studied, two groups were created, one low-level and one high-level group. The evolution of signaling and release indexes were evaluated between baseline and a median follow-up of 60 months, according to the levels of miRNAs expression (low or high).

Statistical analysis

The quantitative data were evaluated as to whether they followed the normal distribution using the Kolmogorov-Smirnov test. The parameters were taken as normally distributed if $p > 0.05$. A comparative analysis of baseline plasma miRNAs levels between the pre-DM or T2DM groups was carried out using the ONE-WAY ANOVA test. Statistical significance for non-normal distribution variables was assessed by non-parametric tests using the Mann Whitney U test. The effect of baseline levels of miRNAs on the insulin resistance, insulin secretion and DI after a median follow-up of 60 months was assessed using the Wilcoxon test. Non-T2DM, pre-DM and incident pre-DM subjects were grouped, and the probability of developing T2DM (Incident-T2DM) was evaluated by Cox regression analysis to test the potential predictive value of the miRNAs studied. The subjects were categorized into tertiles of plasma levels for each miRNA analyzed. Thus, low (T1), intermediate (T2) and high (T3) plasma levels were the three groups defined. The hazard ratio (*HR*) in the Cox regression analysis of each miRNA studied was analyzed by comparing T1 *vs*. T2 and T1 *vs*. T3. $P<0.05$ was considered to be significant. All analyses were adjusted for age, diet, gender, body mass index, glycosylated hemoglobin, HDL-C, triglycerides and waist circumference. Receiver Operating Characteristic curves (ROC) analysis were performed after including the study variables in a logistic binary regression analysis and with the statistic residuals (probabilities). The statistical analysis was carried out using SPSS (now PASW Statistic for Windows (version 21.0) (IBM. Chicago, Illinois, USA)).

RESULTS

Baseline characteristics of patients

In a median follow-up of 60 months, 78 subjects did not develop T2DM or pre-DM (non-T2DM); 239 subjects were pre-DM at baseline, of which 223 subjects maintained pre-DM during the follow-up period (pre-DM); 30 subjects were not pre-DM at baseline but developed pre-DM in the follow-up period (Incident pre-DM). Finally, 107 of the participating subjects developed T2DM (Incident-T2DM) in a median follow-up of 60 months. BMI, waist circumference, total cholesterol, TG, HbA1c, fasting glucose, insulin, glucose after 2 h of OGTT, HOMA-IR and HIRI were all higher in incident-T2DM than pre-DM, incident pre-DM and non-T2DM subjects (all $p < 0.05$). In contrast, ISI, IGI, and DI were lower in incident-T2DM than pre-DM subjects and non-T2DM after a median follow-up of 60 months **(Table 1).**

Baseline levels of circulating miRNAs in the study

We observed that baseline plasma levels of *miR-150* and *miR-30a-5p* were higher and the *miR-15a* and *miR-375* were lower in incident-T2DM compared with non-T2DM subjects, with intermediate values in the pre-DM and incident pre-DM patients (p<0.001; p<0.001; p=0.001 and p=0.044, respectively). We also observed that the plasma levels of *miR-150* were higher in the pre-DM than in the non-T2DM subjects (p=0.020) (**Figure 1**).

Relationship between miRNAs levels and insulin signaling and release indexes

Further, we studied the changes according to the baseline miRNAs levels in the insulin signaling and release indexes (DI, ISI, HIRI and MISI) after 4 years of the follow-up period. The subjects were categorized according to the median of basal plasma levels for each of the four miRNAs studied. We observed that DI decreased after 4 years in subjects with high plasma levels of *miR-150* and *miR-30a-5p* (*p*=0.047 and *p*= 0.007, respectively), as compared with subjects with low plasma levels of these miRNAs. By contrast, no significant differences were observed in *miR-375* and *miR-15a* (**Figure 2**). The DI was lower in patients with high plasma levels of *miR-150* as compared with patients with low plasma levels for this miRNA at 4 years of follow-up (*p*=0.029) (**Figure 2**).

Moreover, the ISI and MISI indexes decreased after the follow-up period in patients with high baseline plasma levels of *miR-150* ($p<0.001$ and $p=0.020$, respectively) when compared with the low plasma levels group (**Table 2**). Likewise, patients with high baseline plasma levels of *miR-375* showed a decrease in the HIRI index after the follow-up period ($p<0.001$) compared to the group with low levels of miRNA **(Table 2).**

Analysis of probability of developing T2DM based on miRNAs levels using the Cox regression model

The risk of developing T2DM based on plasma miRNAs levels was evaluated by Cox regression analysis. We categorized patients by tertiles of the baseline levels for each miRNA: low levels (T1), intermediate levels (T2), and high levels (T3). Our results revealed that patients with high levels (T3) of *miR-150* and *miR-30a-5p* are at a higher risk of developing T2DM than those with low levels (T1) (*miR-150*, *HR* = 4.218; 95% CI: 2.370 – 7.507 and *miR-30a-5p*, *HR* = 2.527; 95% CI: 1.552 – 4.116). In contrast, patients with low levels (T1) of *miR-15a* and *miR-375* are at a higher risk of developing T2DM than those with high levels (T3) (*miR-15a*, *HR* = 3.269; 95% CI: 1.941 – 5.507 and *miR-375*, *HR* = 1.604; 95% CI: 0.957- 2.687) **(Figure 3).**

Comparison between miRNAs-based model and established biomarkers of diabetic risk

Further, we compared the predictive value of our model to the previously established biomarkers and FINDRISC and ADA scores, in order to identify the added predictive power of the miRNAs. We performed a Receiver Operating Characteristic Curves (ROC) analysis, combining our model with the previously established biomarkers and scores. We observed an AUC of 0.714 when clinical variables were used (age, gender, BMI, HDL-C, TG, HbA1c, fasting glucose and fasting insulin), an AUC = 0.759 when we added the OGTT-derived index (DI, MISI, ISI, HIRI and IGI), an AUC = 0.793 when the clinical variables were combined with the miRNAs, an AUC of 0.754 when FINDRISC score was combined with the miRNAs and an AUC of 0.750 when the ADA score was combined with the miRNAs **(Figure 4).**

DISCUSSION

Our study showed that *miR-150* and *miR-30a-5p*, as well as *miR-15a* and *miR-375,* were deregulated in plasma several years before the diagnosis of T2DM. Specifically, we demonstrated for the first time that the baseline plasma levels of *miR-150* and *miR-30a-5p* were higher and *miR-15a* and *miR-375* lower in incident-T2DM subjects compared with non-T2DM after a median follow-up of 60 months, with intermediate levels in incident pre-DM and pre-DM subjects. Moreover, our study showed that a higher risk of developing T2DM is associated with low baseline plasma levels of *miR-15a* and *miR-375* and high baseline plasma levels of *miR-150* and *miR-30a-5p*. Our study also revealed the potential use of the circulating miRNAs-based tools for predicting type 2 diabetes development in clinical practice, showing a small but higher adding predictive value to the usual clinical variables used such as age, gender, BMI, HDL-C, TG, HbA1c, fasting glucose and fasting insulin, than the predictive value added by indices derived from OGTT, such as DI, MISI, ISI, HIRI and IGI to these variables.

Circulating miRNAs in human biofluids such as blood [43] has led to their use as non-invasive biomarkers for multiple pathologies including CVD and T2DM [44-46]. This is based on the evidence that miRNAs play a major role in different mechanisms involved in T2DM development, such as insulin production, secretion and action, which supports their use as biomarkers for T2DM diagnosis [47]. However, the alteration in the miRNA pattern may precede or appear at early stages of diabetes, or, may be a consequence of the onset of diabetes [48].

In this study, we have shown that the plasma levels of four miRNAs are deregulated in prediabetic states, several years preceding the development of T2DM, suggesting their potential use in the development of circulating miRNAs-based tools for

T2DM prediction. When comparing the AUC of the ROC curves, we observed an improvement in the predictive capacity of the parameters used in clinical practice when the miRNAs were added to the model. However, this improvement was slightly higher than the improvement observed when OGTT-derived indexes are added to the clinical variables. Nevertheless, these findings suggest a potential use of miRNAs in clinical practice as they can be determined by a single blood collection, whereas OGTT requires continuous blood sampling (every half-hour for 2 hours) in order to calculate the indexes.

In addition, the combined use of the FINDRISC and ADA scores with miRNAs yielded lower predictive power, probably because of the fact that both scores include parameters not specific for T2DM and/or that they are self-reported, which diminishes their reliability and specificity for the prediction of T2DM status.

In fact, Cox proportional hazards regression analysis showed that patients with high levels of *miR-15a* and *miR-375* are at low risk of developing T2DM (HR 3.269 and 1.604, respectively), whereas patients with high levels of *miR-150* and *miR-30a-5p* are at high risk of developing T2DM (HR 4.218 and 2.527, respectively). The idea of the usefulness of miRNAs plasma levels to assess the risk of T2DM development is supported by other studies, in which alterations in plasma miRNAs levels have been observed before the development of T2DM [18, 19].

However, whereas the study by Zampetaki et al. is a case-control study in which matched controls were selected, our incidence study included all subjects from the CORDIOPREV cohort without T2DM at baseline and we used all the non-T2DM patients after the follow-up period as controls.

Whereas our study evaluates the incidence of T2DM, the Zampetaki et al. study compares the differences between groups. The two study designs have their pros and cons: for example, in the case-control study, the recruitment of controls could be prone to selection bias—that is, the controls are systematically different from the population they are meant to represent. However, careful matching, aimed at eliminating any possible confounding factors, leads to efficiency in the study. Otherwise, incidence studies are usually the preferred approach for studying the causes of disease, because they use all the available information on the source population over the risk period. However, they are often extremely costly in terms of time and resources, and equivalent results can be achieved more efficiently by using an incidence case–control study design. Although there is a difference in design, both studies demonstrate the potential of miRNAs as biomarkers of T2DM.

The study by Willeit et al. and the present work are incidence studies in which statistical and predictive analysis can be performed to evaluate the risk of T2DM development. Nevertheless, in contrast to the study by Willeit et al. [18], which focused on *miR-122* to evaluate the risk of T2DM, our study included four miRNAs, which were combined with several clinical variables, such as age, gender, BMI, HDL-C, TG, HbA1c, fasting glucose and fasting insulin; this allowed us to build predictive models by adding miRNAs, which increased the predictive power of the traditional clinical parameters.

In addition, in our study, we followed ADA diagnosis criteria, whereas the studies by Zampetaki et al. and by Willeit et al. followed the World Health Organization guidelines, which do not take into account plasma levels of glycosylated hemoglobin as diagnosis criteria for diabetes, but it was used for the clinical validation of new cases of diabetes. Although these two methods are not very different, when the World Health Organization guidelines are followed, new cases of diabetes diagnosed by glycosylated hemoglobin are not detected.

Overall, *miR-150*, with a potential role in the main tissues for glucose homeostasis, and *miR-15a*, involved in insulin production and secretion, showed better risk assessment than *miR-30a-5p* and *miR-375.* However, analyses based on indexes of insulin signaling and release have shown that in patients with high circulating levels of *miR-150,* DI, ISI and MISI decreased during the follow-up period.

In fact, *miR-150* is highly expressed in the main tissues for glucose homeostasis such as adipose, skeletal muscle and liver [13]. Moreover, this miRNA might come from haematopoetic cells, and the increase in *miR-150* could be a secondary phenomenon due to low-grade inflammation. This idea is supported by the involvement of this miRNA in the activation of B cells and other immune cells in adipose tissue, which, in turn, increases the insulin resistance of this tissue [37]. In addition, our study showed a relationship between *miR-150* plasma levels and the indexes derived from OGTT, which are related with the insulin signaling and release. However, further studies are needed to clarify which tissues are responsible for releasing *miR-150* into the blood stream and the mechanisms that regulate this miRNA.

In contrast, *miR-15a* positively regulates insulin biosynthesis by inhibiting endogenous UCP-2 (uncoupling protein-2) expression, leading to higher ATP levels in islets improving glucose-stimulated insulin secretion (GSIS). This increase in intracellular ATP closes the ATP-sensitive potassium channel, causing plasma membrane depolarization, the influx of Ca^{2+} and GSIS [35, 49]. Thus, the lower levels of *miR-15a* that we observed in the patients who develop pre-diabetes and T2DM suggest that an impairment of stimuli for insulin secretion occurs before the development of T2DM. Moreover, our results are in line with previous studies, which have shown that circulating *miR-15a* levels decreased significantly before the onset of type 2 diabetes mellitus [14, 19].

Regarding miRNAs functions, the potential role of *miR-150* on insulin resistance in obesity by controlling adipose tissue inflammation seems to have a higher physiological impact than the change in insulin signaling by *miR-15a,* as suggested by the close relationship found between *miR-150* and the DI analyzed in our study. This idea is supported by the fact that the high plasma levels of *miR-30a-5p*, which have previously been shown to modulate the beta cell function, were both associated with a decrease in DI. In fact, the overexpression of *miR-30a-5p* has been linked to the suppression of Beta2/NeuroD, which plays a key role in the regulation of insulin secretion and pancreatic beta cell dysfunction during glucotoxicity [12]. *Beta2/NeuroD* is a transcription factor that binds to the E element of the insulin gene [50] and modulates the K^+ channels to regulate insulin secretion [51].

Studies in animal models have shown the relationship of *miR-375* with the development of T2DM. In fact, *miR375KO* mice has a mass reductions in beta-cells and an increase in the number of α cells, leading to islet instability [34]. Moreover, it has also been shown that the overexpression of *miR-375* in INS-E cells inhibits insulin gene expression in response to glucose, by down-regulation of the PDK1 gene, leading also to PI3K pathway inhibition [52]. In this sense, more research is needed to clarify the relationship between *miR-375* and the development of T2DM.

Certain limitations of the current study must be mentioned. The first limitation lies in the fact that we performed a focused bibliographic search of miRNAs associated with insulin sensitivity, insulin secretion and growth and proliferation of beta-cell, and other potential miRNAs T2DM-related that has not been described, were not included in our study. Another limitation lies in the fact that we analyzed plasma levels of miRNAs, and this approach, while suitable for assessing disease risk, may not accurately reflect the involvement and directionality in the interactions of these miRNAs in processes occurring at cellular levels. Moreover, the prevention of T2DM was not the primary endpoint of the CORDIOPREV trial but was a secondary analysis conducted in the subgroup of cardiovascular patients without T2DM at baseline (CORDIOPREV-DIAB study). The study included a large number of older patients with acute myocardial infarction, which limits our findings to people with these characteristics and precludes generalization to healthy people.

In conclusion, our study showed that deregulated plasma levels of *miR-150*, *miR-30a-5p*, *miR-15a*, and *miR-375* were observed years before the onset of T2DM and pre-DM and could be used to evaluate the risk of developing the disease, which may improve prediction and prevention among individuals at high risk of T2DM.

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AUTHOR CONTRIBUTION: The authors' responsibilities were as follows: J-LR, R-ZOA and L-MJ, conceived and designed the experiments. A-DJF, D-LJ, P-MP and L-MJ participated in the recruitment and carried out the clinical and nutritional control of the volunteers. J-LR, C-A and R-ZOA performed the experiments and collected the data. J-LR, C-A, A-DJF, R-ZOA and L-MJ analyzed and interpreted the data. J-LR, C-A, R-ZOA and L-MJ drafted the manuscript. L-MJ conceived and designed the study. vO-B, L-RM, O-JM and L-MJ provided critical revision of the paper for the important intellectual content. R-ZOA, D-LJ, P-MP and L-MJ had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. All the authors were involved in writing the paper and gave their final approval to the submitted and published versions. None of the authors has any conflict of interest that could affect the performance of the work or the interpretation of the data.

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LEGENDS FOR FIGURES

FIGURE 1. Baseline levels of miRNAs studied according to Pre-DM or T2DM status at median follow-up of 60 months. Values expressed as mean ± standard error. Variables were calculated using ONE-WAY ANOVA analysis through SPSS (now PASW Statistic for Windows (version 21.0) (IBM. Chicago, Illinois)). Subjects did not develop T2DM or pre-DM (non-T2DM); Subjects maintained pre-DM during the follow-up period (pre-DM); Subjects not pre-DM at baseline but developed pre-DM in the follow-up period (Incident pre-DM); Subjects developed T2DM (Incident-T2DM). Significance was assessed by non-parametric tests using the U Mann-Whitney test. * $p<0.05$.

FIGURE 2. Disposition index changes according to median levels of miRNAs. Values expressed as mean ± standard error. Variables were calculated by Wilcoxon test using SPSS (now PASW Statistic for Windows (version 21.0) (IBM. Chicago, Illinois)). Dotted gray line shows subjects with low levels of miRNA at baseline and continuous black line shows subjects with high levels of miRNA at baseline. § $p < 0.05$ baseline vs 4y. # $p <0.05$ Low expression levels *vs* high expression levels groups.

FIGURE 3. Disease-free survival by COX proportional hazards regression analysis of miRNAs. Subjects were divided into low (T1), intermediate (T2) and high (T3) baseline levels of miRNAs. Dotted black line represents subjects from T1, dotted gray line represents subjects from T2 and continuous black line represents subjects from T3. Analyses were adjusted by age, diet, gender, body mass index, glycosylated haemoglobin, HDL, triglycerides and waist circumference.

FIGURE 4. Receiver Operating Curves (ROC) analysis. A) ROC model including clinical variables (Age, gender, BMI, HDL-C, TG, HbA1c, fasting glucose and fasting insulin); B) ROC model including clinical variables and OGTT-derived indexes (DI, MISI, ISI, HIRI and IGI); C) ROC model including clinical variables and studied miRNAs; D) ROC model including studied miRNAs and FINDRISC score; and E) ROC model including studied miRNAs and ADA score.

The analyses were performed using SPSS (now PASW Statistic for Windows (version 21.0) (IBM. Chicago, Illinois)).

Table 1. Baseline characteristics of different groups of patients included in the study.

	Non-T2DM	*Pre-DM*	*Incident Pre-DM*	*Incident-T2DM*	*p-value*
N	78	223	30	107	
Age (years)	**53 ± 1**	**59 ± 1[a]**	**57 ± 2**	**59 ± 1[c]**	**<0.001***
BMI (kg/m²)	**28.9 ± 0.4**	**30.3 ± 0.3**	**29.3 ± 1.0**	**31.4 ± 0.5[c]**	**0.001***
Waist circumference (cm)	**99.1 ± 1.1**	**102.8 ± 0.7[a]**	**99.1 ± 2.3**	**105.3 ± 1.1[c]**	**<0.001***
Total cholesterol (mg/dl)	**162 ± 3**	**160 ± 2**	**147 ± 6**	**165 ± 3**	**0.042***
c-HDL (mg/dl)	46.2 ± 1.2	44.0 ± 0.7	43.2 ± 2.0	43.5 ± 1.0	0.236
TG (mg/dl)	**125 ± 9**	**120 ± 4**	**94.5 ± 8.4**	**133 ± 7**	**0.011***
c-LDL (mg/dl)	91.5 ± 2.8	91.7 ± 1.7	82.8 ± 3.6	93.4 ± 2.7	0.301
hs-CRP (mg/L)	2.37 ± 0.44	2.57 ± 0.24	1.98 ± 0.36	2.88 ± 0.29	0.066
HbA1c (%)	**5.66 ± 0.04**	**5.97 ± 0.02 [a]**	**5.43 ± 0.04 [b]**	**6.03 ± 0.03 [c]**	**<0.001***
Glucose (mg/dl)	**88.8 ± 1.1**	**94.0 ± 0.7[a]**	**88.1 ± 1.2**	**96.2 ± 1.0 [c]**	**<0.001***
Insulin (mU/l)	**8.15 ± 0.69**	**8.57 ± 0.39**	**7.83 ± 0.88**	**10.51 ± 0.66**	**0.017***
Glucose 2 h after OGTT (mg/dl)	**110 ± 4**	**125 ± 2 [a]**	**113 ± 4**	**135 ± 3 [c]**	**<0.001***
ISI	**5.02 ± 0.30**	**4.02 ± 0.18 [a]**	**4.36 ± 0.45**	**3.27 ± 0.19 [c]**	**<0.001***
HOMA-IR	**2.22 ± 0.17**	**2.71 ± 0.12**	**2.26 ± 0.19**	**3.23 ± 0.22 [c]**	**0.001***
HIRI	**912 ± 71**	**1102 ± 46**	**932 ± 77**	**1313 ± 90 [c]**	**0.002***
IGI	**1.04 ± 0.09**	**1.16 ± 0.08**	**1.42 ± 0.19**	**0.88 ± 0.07**	**0.038***
DI	**1.14 ± 0.06**	**0.93 ± 0.04 [a]**	**1.27 ± 0.11**	**0.77 ± 0.04 [c]**	**<0.001***
MISI (x 10^2)	2.48 ± 0.28	2.01 ± 0.14	1.54 ± 0.19	1.89 ± 0.16	0.125

Subjects were classified according to pre-DM and T2DM-development status after a follow-up median of 60 months. Values expressed as mean ± standard error. ***BMI***, Body mass index; ***c-HDL***, High density lipoprotein; ***c- LDL***, Low density lipoprotein; ***TG***, Triglycerides; ***hs-CRP***, High sensitivity C-reactive protein; ***HbA1c***, Glycosylated hemoglobin; ***HIRI***, Hepatic insulin resistance index; ***MISI***, Muscle insulin sensitivity index; ***ISI***, Insulin sensitivity index; ***IGI***, Insulinogenic index; ***DI***, Disposition index; ***HOMA-IR***, Homeostasis model assessment- insulin resistance; * $p < 0.05$. Variables were calculated by ONE-WAY ANOVA analysis using SPSS (now PASW Statistic for Windows (version 21.0)) (IBM. Chicago, Illinois). Subjects did not develop T2DM or pre-DM (**non-T2DM**); Subjects maintained pre-DM during the follow-up period (**pre-DM**); Subjects not pre-DM at baseline but developed pre-DM in the follow-up period (**Incident pre-DM**); Subjects developed T2DM (Incident-T2DM). Post Hoc analysis: [a] p< 0.05 non-T2DM *vs* pre-DM subjects, [b] p< 0.05 non-T2DM *vs* incident pre-DM subjects; [c] p< 0.05 non-T2DM *vs* incident-T2DM subjects.

		miR-150 levels		***miR-30a-5p levels***		***miR-15a levels***		***miR-375 levels***	
		low	**high**	**low**	**high**	**Low**	**high**	**low**	**high**
DI	**0y**	**0.95 ± 0.03**	**0.93 ± 0.03**	**0.95 ± 0.03**	**0.92 ± 0.03**	0.91 ± 0.04	0.96 ± 0.03	0.94 ± 0.03	0.93 ± 0.03
	4y	**0.94 ± 0.04**	**0.85 ± 0.03***	**0.94 ± 0.03**	**0.85 ± 0.04***	0.89 ± 0.04	0.89 ± 0.04	0.87 ± 0.04	0.92 ± 0.04
HIRI	**0y**	1069 ± 42	1040 ± 41	1020 ± 41	1088 ± 41	1046 ± 41	1062 ± 41	**1027 ± 41**	**1080 ± 41**
	4y	958 ± 64	967 ± 61	947 ± 62	978 ± 62	986 ± 62	940 ± 62	**1012 ± 62**	**914 ± 62***
MISI	**0y**	**1.81 ± 0.14**	**2.19 ± 0.14**	2.15 ± 0.14	1.86 ± 0.15	2.31 ± 0.14	1.73 ± 0.14	1.99 ± 0.14	2.03 ± 0.14
($x10^2$)	**4y**	**2.02 ± 0.15**	**1.79 ± 0.14***	1.82 ± 0.15	1.98 ± 0.15	2.17 ± 0.15	1.65 ± 0.14	1.97 ± 0.15	1.83 ± 0.15
ISI	**0y**	**3.84 ± 0.18**	**4.10 ± 0.17**	3.99 ± 0.17	3.97 ± 0.17	4.13 ± 0.17	3.84 ± 0.17	3.96 ± 0.17	4.01 ± 0.17
	4y	**3.98 ± 0.19**	**3.71 ± 0.18***	3.83 ± 0.18	3.84 ± 0.18	4.20 ± 0.18	3.50 ± 0.18	3.86 ± 0.18	3.81 ± 0.18

Table 2. T2DM-related indexes between baseline and 4 years of follow-up divided by the median of baseline circulating miRNAs. Values are expressed as mean ± SEM and were obtained by ANOVA for repeated measures. **Low**: Low circulating levels of miRNAs; **High**: High circulating levels of miRNAs. Statistical significance was evaluated by Wilcoxon test through SPSS (now PASW Statistic for Windows (version 21.0) (IBM. Chicago, Illinois)). * $p < 0.05$.

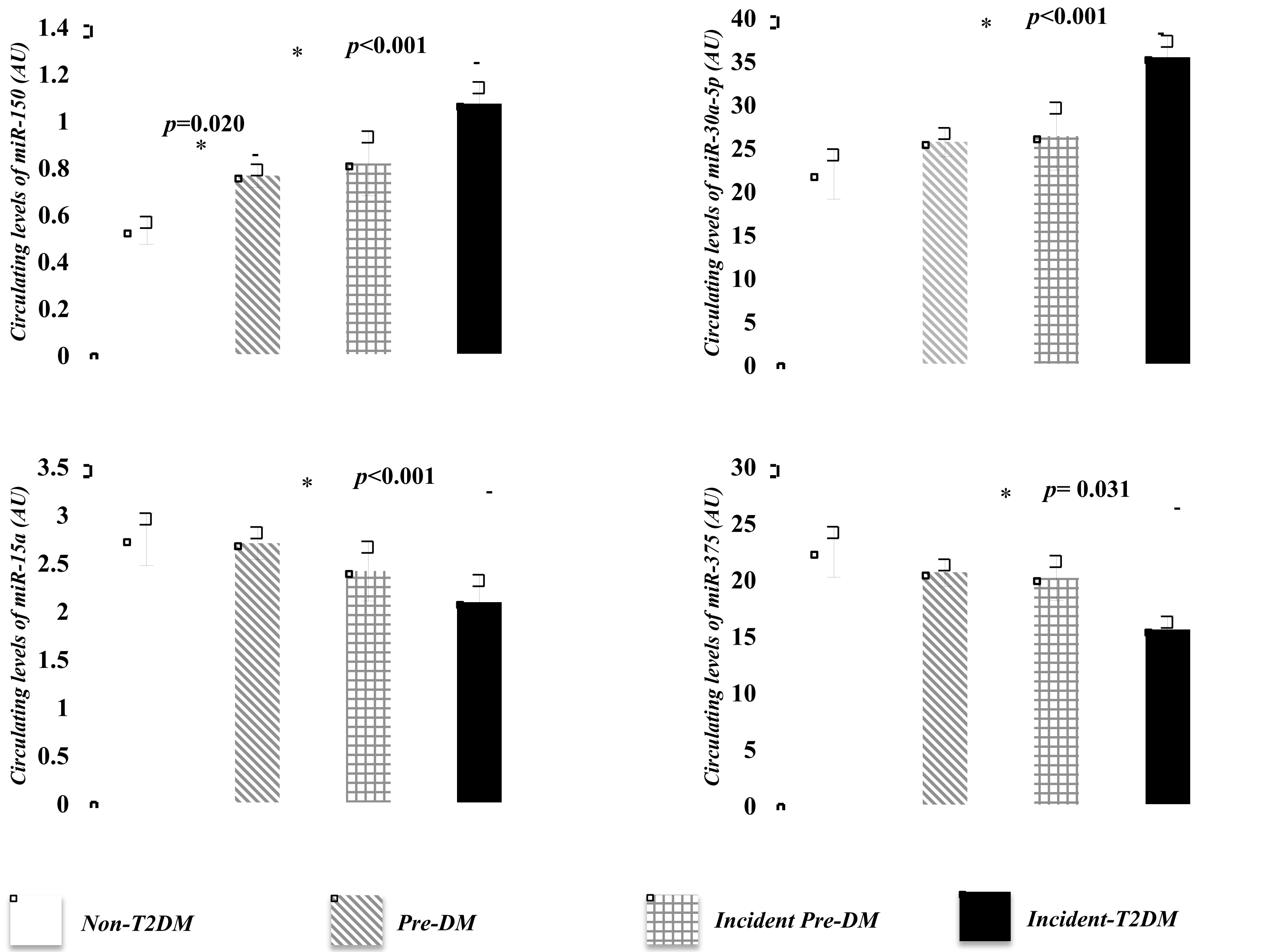

FIGURE 1. Baseline levels of miRNAs studied according to Pre-DM or T2DM status at median follow-up of 60 months.

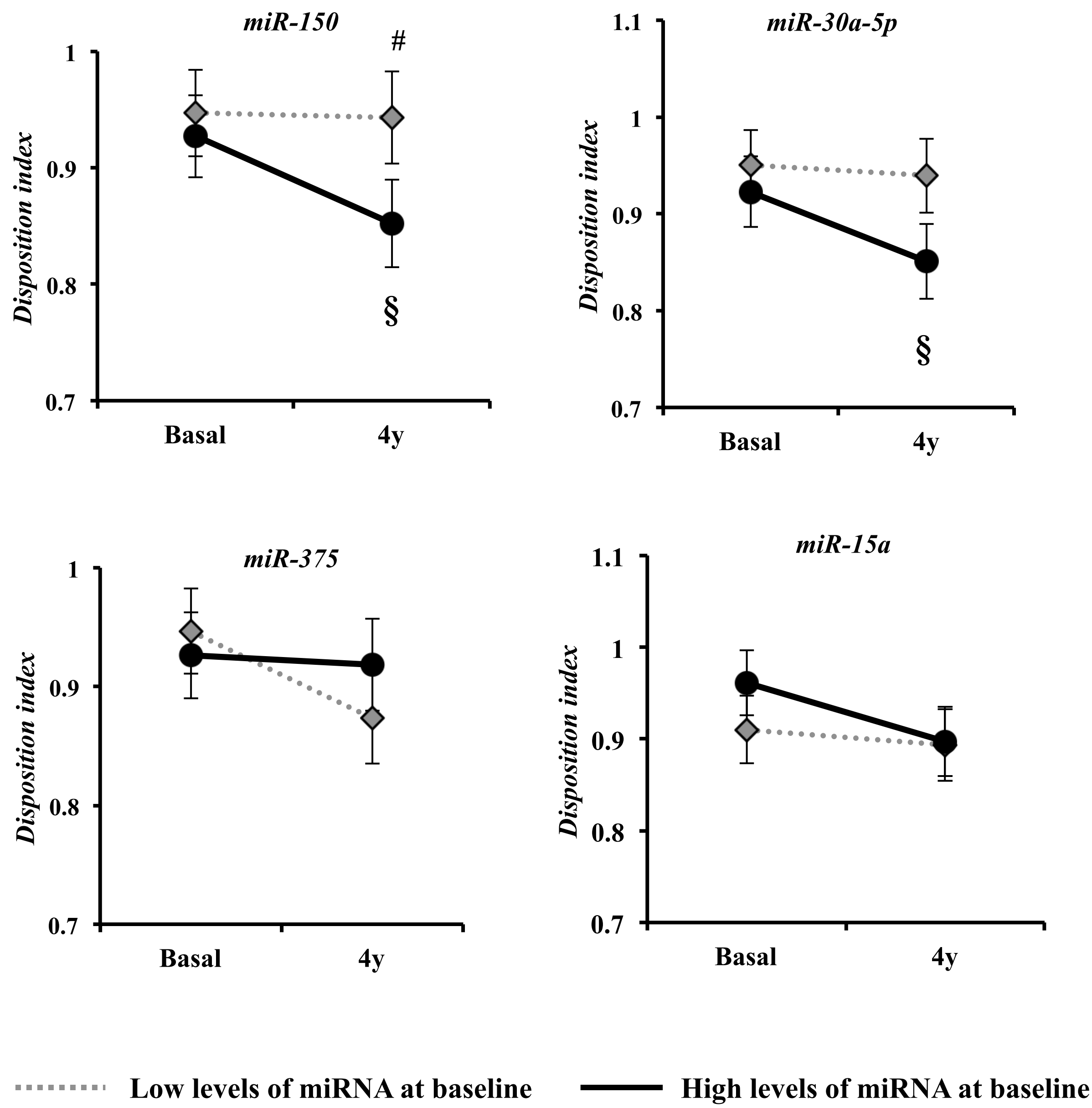

FIGURE 2. Disposition index changes according to median levels of miRNAs.

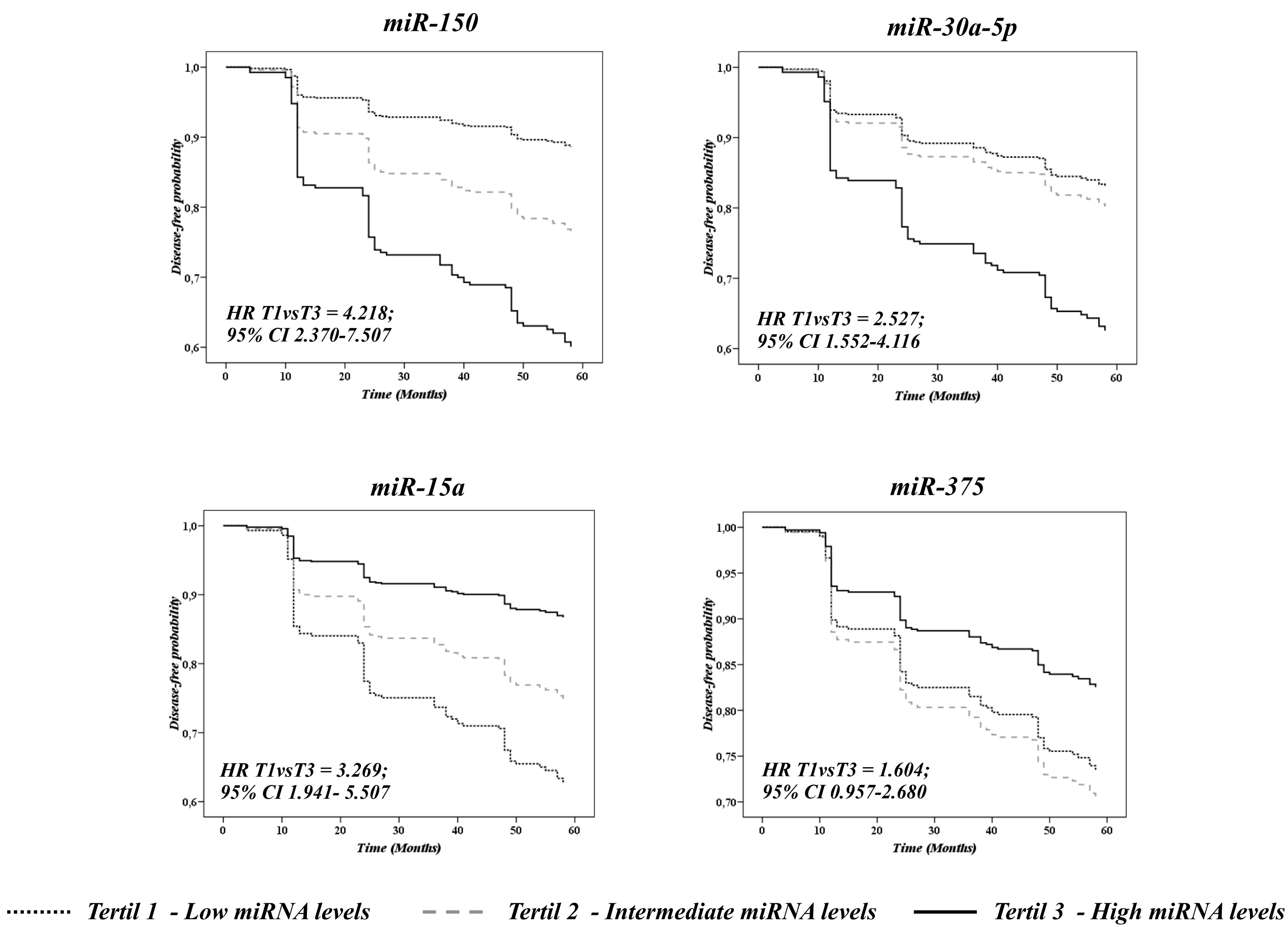

FIGURE 3. Disease-free probability by COX proportional regression analysis of miRNAs.

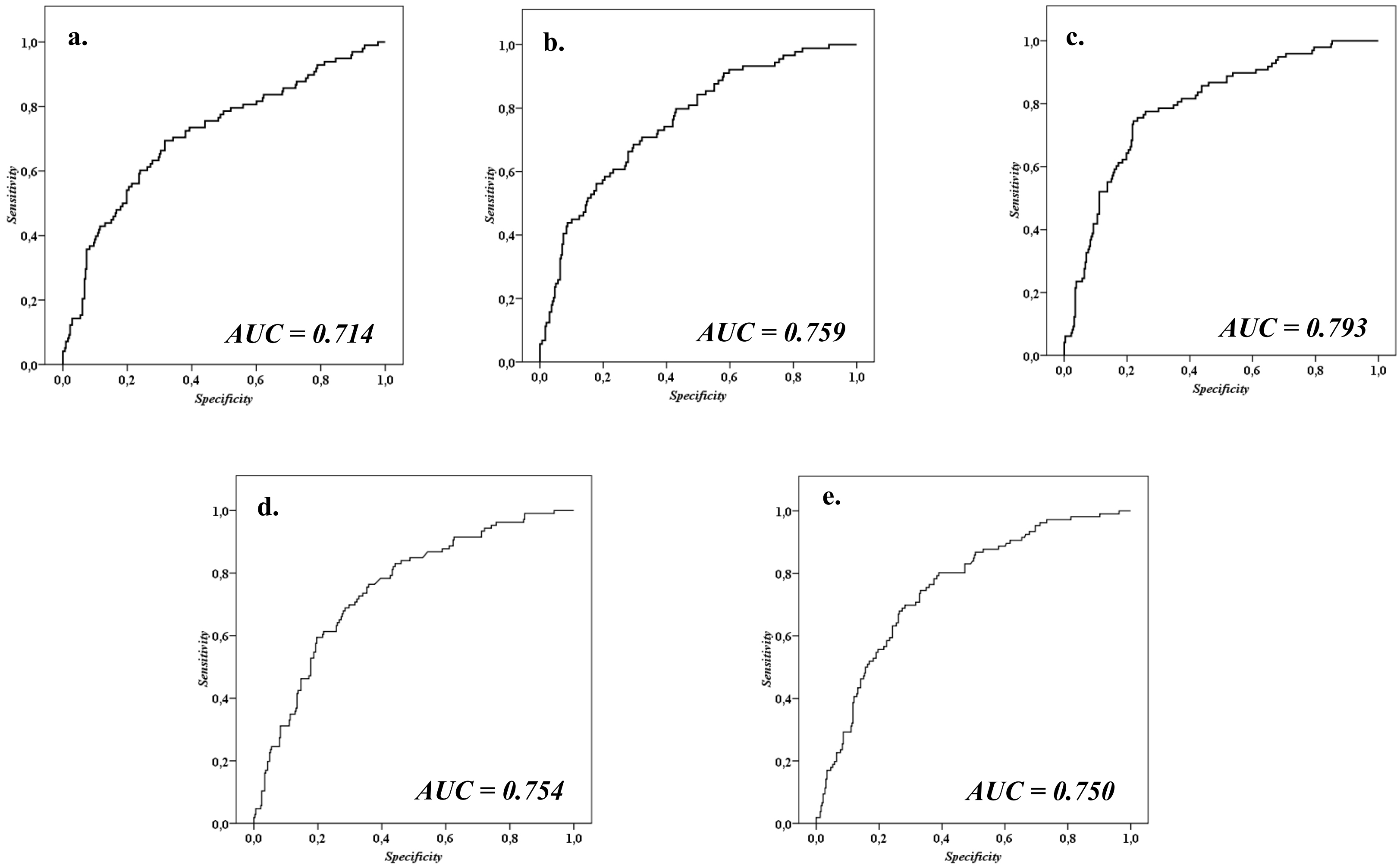

FIGURE 4. Receiver Operating Curves (ROC) analysis.

VI. OTHER APPROACH

Metabolomic approach for the identification of subjects at risk of developing Type 2 Diabetes Mellitus

Metabolomics studies the significant changes in the global pattern of metabolites in biological samples to identify potential biomarkers of disease, as well as to assess the efficacy of a therapy or a nutritional intervention [323]. There are different factors that influence in the metabolic profile of biological samples, such as race, gender, age, stress or the intestinal microbiota. Changes in the abundance of metabolites could precede development of the disease, suggesting their use as biomarkers of disease prediction [324].

The marked increase in the incidence of type 2 diabetes mellitus (T2DM) worldwide is a serious public health problem and an economic burden on the global economy [325]. The identification of people at high risk of developing this disease is a key element in designing prevention strategies.

Based on this background, our aim was to evaluate the differences in the metabolomic profiles between subjects who develop and do not develop T2DM after 3 years of follow-up in order to identify metabolites with potential value as predictive biomarkers of the development of this disease.

MATERIAL AND METHODS

Study subjects

We selected a group of 52 participants from the CORDIOPREV study without T2DM at baseline, 26 of whom developed the disease (Incident-T2DM) according to ADA criteria and another 26 matched subjects, who did not develop it (non-T2DM) after 3 years of follow-up, served as controls. For this pilot study, the choice of Incident-T2DM subjects was based on the pronounced increased levels of fasting glucose, glucose after 2 hours of OGTT and glycosylated hemoglobin (ADA criteria) after the follow-up period.

Methodology of the metabolic fat challenge

A fat overload was performed at the beginning of the study. Before starting the test, the patients had fasted (food/drugs) for 12 h and were asked to refrain from smoking during the fasting period and from alcohol intake during the preceding 7 days. They were also asked to avoid strenuous physical activity the day before the test was carried out.

The patients arrived at the health center at 8.00 a.m. A fasting blood sample was taken, and immediately afterwards, the patients ingested a weight-adjusted meal (0.7-g fat and 5-mg cholesterol per kg body weight) with 12% saturated fatty acids (SFAs), 10% polyunsaturated fatty acids (PUFA), 43% monounsaturated fatty acids (MUFAs), 10% protein and 25% carbohydrates (CHO). The meal was prepared by nutritionists using olive oil, skimmed milk, white bread, cooked egg yolks and tomatoes. After the meal, the volunteers rested and consumed no food for 4 h, but were allowed to drink water. In addition to the fasting sample, blood was extracted for biochemical testing every hour during the 4 h after the meal, according to the established protocols [326].

Metabolomic Analysis

Sample Preparation

The metabolomics studies were carried out by duplicate sampling at the beginning of the study (baseline) in the fasting state and after 4 hours of fat overload (postprandial state). Plasma samples (60 µL) were deproteinized with 200µL of MeOH:ACN (3:1). The mixture was vortexed for 1 min and subsequently cooled at −20 °C for 3 min. The resulting precipitate was separated by centrifugation at 14000×g for 15 min at 4 °C and the supernatant phase was isolated. This phase was dried by evaporation.

For analysis by LCQTOF, the resulting residues were reconstituted with 60 µL of MeOH:ACN (3:1) and shaken in a vortex for 60 s. For derivatization, the resulting residues were reconstituted with 20 µL of methoxyamine in pyridine (20mg/mL) and maintained at 30 °C for 90 min, and later, 180 µL of a 98:2 (v/v) BSTFA–TMCS mixture was added to the reconstituted analytical sample, shaken for 30 s and incubated at 37 °C for 60 min.

LC–QTOF MS/MS Analysis

Chromatographic separation was performed by using a Poroshell 120 EC-C18 column (50 mm × 2.1 mm i.d., 2.7 µm particle size) which was thermostated at 25 °C and protected using an EC-C18 precolumn (4.5 × 2.1 mm i.d., 2.7 µm particle size) from Agilent Technologies. The mobile phases were 95:5 water:ACN (phase A) and 95:5 ACN:water (phase B), both containing 0.1% (v/v) formic acid and 5 mM ammonium acetate as ionization agents. The LC pump was programmed at a flow rate of 0.4 mL min–1 and the elution gradient was as follows: from min 2 to 11, the percentage of phase B was modified from 0% to 100% and then, the final percentage was hold for 6 min. A post-time of 7 min was used to regain the initial conditions for the next analysis. Thus, the total analysis time per sample was 34 min (including postprocessing). The injected volume was 5 µL, and the injector needle was washed 10 times between injections with 80% MeOH. Also, the needle seat back was flushed with 80% MeOH at 4 mL min–1 for 12 s to avoid cross contamination. The autosampler was kept at 4 °C to increase sample stability. The settings of the electrospray ionization source, which was operated in the negative and positive ionization modes, were as follows: capillary voltage ±3.5 kV, Q1 voltage 130 V, N2 pressure in the nebulizer 35 psi; N2 flow rate and temperature as drying gas 10 L min–1 and 325 °C, respectively. MS/MS data were acquired in both polarities, using the centroid mode at a rate of 2.5 spectra s–1 in the extended dynamic range mode (2 GHz). Accurate mass spectra in MS scan were acquired in the m/z range 40–1100, and in MS/MS mode in the m/z range 30–1100. The instrument gave typical resolution 18,000 Full Width at Half Maximum (FWHM) at m/z 118.0862 and 35,000 FWHM at m/z 922.0098. The instrument was calibrated and tuned as recommended by the manufacturer. To assure the desired mass resolution, continuous internal calibration was performed during analyses by using the signals at m/z 121.0509 (protonated purine) and m/z 922.0098 [protonatedhexakis(1H,1H,3H-tetrafluoropropoxy) phosphazine or HP-921] in the positive ion mode; while in the negative ion mode, ions with m/z 119.0362 (proton abstracted purine) and m/z 966.0007 (formate adduct) were used. The collision energy was set at 20 V for the whole run. The analytical samples were injected in auto MS/MS acquisition mode to obtain information from fragmentation of the target compounds. The maximum number of precursors selected per cycle was set at 2, with an exclusion window of 0.1 min after 2 consecutive selections of the same precursor.

GC–TOF/MS Analysis

GC–TOF/MS analyses were performed by EI ionization mode at 70 eV. Chromatographic separation was carried out with a fused silica DB-5MS-UI 30 m × 0.25 mm i.d, × 0.25 µm film thickness capillary column. The GC oven temperature program started at 60 °C (1 min held), followed by a temperature ramp of 10 °C min−1 to final 300 °C (2 min held). Post-run time was programmed for 4 min up to 310 °C to assure complete elution of the injected sample. Pulsed splitless injections of 1 µL of sample were carried out at 250 °C and ultrapure grade helium was used as carrier gas at 1.0 mL min^{-1} flow rate. The interface, ion source and quadrupole temperatures were set at 280, 300 and 200 °C, respectively. A solvent delay of 5 min was used to prevent damage in the ion source filament. The TOF detector was operated at 5 spectra s^{-1} in the mass range m/z 50–550 and the resolution was 8500 full width half maximum (FWHM) at m/z 501.9706. A daily mass calibration was performed with PFTBA.

Data processing, identification of metabolites

Data processing and identification of metabolites by LC–QTOF MS/MS

MassHunter Workstation software (version B7.00 Qualitative Analysis, Agilent Technologies, Santa Clara, CA, USA) was used to process all data obtained by LC–QTOF in data-dependent acquisition MSMS mode. Treatment of raw data files started by extraction of potential molecular features (MFs) with the suited algorithm included in the software. For this purpose, the extraction algorithm considered all ions exceeding 1500 counts for both polarities with a single charge state for the obtained chromatograms. These cut-off values were established taking into account the chromatographic background noise. Additionally, the isotopic distribution to consider MFs as valid should be defined by two or more ions (with a peak spacing tolerance of m/z 0.0025, plus 5.0 ppm in mass accuracy). Ions and adducts formation in the positive (+H, +Na, +K, $+NH_4$) and negative ionization (−H, +HCOO, +Cl) modes, as well as neutral loss by dehydration were included to identify features corresponding to the same potential metabolite.

Identification of metabolites was supported on MS and MS/MS information that was searched in the METLIN MS and MS/MS databases (http://metlin.scripps.edu), the Human Metabolome Database (HMDB, 3.6 version) and the LIPID MAPS website ((http://www.lipidmaps.org), using in all cases the MFs obtained in the previous step. A database with all identified metabolites was used to perform a targeted compound extraction analysis using a tolerance window of 0.8 min and 5 ppm mass accuracy. This step was performed with Profinder Analysis (version B8.00, Agilent Technologies, Santa Clara, CA, USA). A table with the peak area of all identified compounds in the different samples injected was obtained as a result.

Data processing and identification of metabolites by GC–TOF/MS

Unknown Analysis software (version 7.0, Agilent Technologies, Santa Clara, CA, USA) was used to unzip all data files obtained by GC–TOF/MS in full scan mode. Then, MassHunter software was used to process GC–TOF/MS data files. Treatment of raw data files started by deconvolution of chromatograms to obtain a list of MFs considered as potential compounds defined by the m/z value of one representative ion for each chromatographic peak and its RT. For this purpose, the deconvolution algorithm was applied to each sample by considering all ions exceeding 1500 counts for the absolute height parameter, the accuracy error at 50 ppm and the window size factor at 150 units. The list of MFs obtained for each analysis was exported as data files in compound exchange format (.cef files). Tentative identification of compounds was performed by searching each mass spectrum in the NIST database (version 11) using the RI value. The identification was firstly carried out by searching MS spectra on the NIST database. Only those identifications with a match factor and a reverse match factor higher than 700 were considered as valid. The RI values included in the NIST database were also taken into account to support identifications. An RI calibration model was built by plotting the retention times obtained by analysis of the alkane standard mixture (C10 to C40 with an even number of carbons) with the chromatographic method used in this research and the RI values provided for each alkane by the NIST database. Then, the RI value was experimentally estimated for each identified compound by using the retention time and the calibration equation. The requirement to accept NIST database identifications was that the difference between the experimental RI and the theoretical value provided by the NIST for each target compound should be below 100 units. The NIST database does not contain high resolution MS information as provided by the TOF detector. For this reason, a third step was included to validate identification of each compound by using high resolution MS. Thus, the molecular formula for the $[M]+$ ion and the most intense fragments for each molecular feature (MF) should fit the NIST 11 identification by setting a cut-off value in mass accuracy of 5 ppm. A table with the peak area of all identified compounds in the different samples injected was obtained as a result.

Principal Component Analysis (PCA)

Prior to the PCA, the data were normalized into a dataset suitable for analysis. Applying the procedures of mean-centering and unit variance (UV) –scaling, the data of the metabolite plasma levels were processed using SIMCA-P+ (version 14.0.0.1359; Umetrics, Umea, Sweden). Principal component analysis (PCA) was performed with the dataset and the score plots were visually inspected for detection of patterns and outliers. Partial least squares discriminant analysis (PLS-DA) and Orthogonal partial least squares discriminant analysis (OPLS-DA) were used to compare metabolites profiles at baseline, in the fasting and the postprandial state to analyse the differences between Incident-T2DM and non-T2DM at the beginning of the study. PLS-DA and OPLS-DA validation was performed by cross-validation (CV) method in the SIMCA-P software by default setting which includes a procedure of 7-fold cross-validation where the dataset is split into 7 different subsets [5]. The quality of the models obtained by PLS-DA and OPLS-DA was assessed by interrogation of the R2 and Q2 parameters [5, 6]. Next, we selected those metabolites with higher discriminatory power between groups from the Variables Importance Projection (VIP) score obtained in the PLS-DA and OPLS-DA models. Metabolites with a VIP score >1.5 were considered important for differentiating between groups.

Receiver Operating Characteristic (ROC) Analysis

Analysis of ROC were carried out to know the predictive potential of the identified metabolites on the T2DM development. For that, we estimated the accuracy, specificity and sensitivity of the variables for Incident-T2DM and non-T2DM differentiation. We used Metaboanalyst 3.0 to analyze the discriminating accumulative effect of all the variables studied. Data normalization was performed using the auto-scaling method, based on mean-centered and divided by the standard deviation of each variable. Next, Multivariate ROC curve analysis was conducted based on the random forest algorithm. We report the area under the curve (AUC) and 95% CI for each combination of variables [7, 8] and we considered $p < 0.05$ as significant.

RESULTS

Baseline characteristics of subjects

At baseline, we observed lower insulinogenic index (IGI) values in the Incident-T2DM than in the non-T2DM group (all, $p<0.05$). No significant differences were observed between the two study groups in the other parameters analyzed (**Table 1**).

Table 1. *Baseline characteristics of subjects who did not develop T2DM (Non-T2DM) vs subjects who developed T2DM (Incident-T2DM) after 3 years of follow-up*

Variables	*Non-T2DM*	*Incident-T2DM*	*p- value*
N	26	26	
Age (years)	63 ± 5.4	62 ± 7.7	0.887
Waist circumference (cm)	104.3 ± 2.2	103.9 ± 1.8	0.869
BMI (kg/m^2)	30.6 ± 0.6	30.2 ± 0.7	0.720
TG (mg/dl)	108.7 ±11.9	122.6 ±11.2	0.399
Total cholesterol (mg/dl)	161.9 ±6.6	161.6 ±7.1	0.975
c-LDL (mg/dl)	94.1 ±4.9	94.5 ±5.5	0.964
c-HDL (mg/dl)	46.8 ± 2.1	42.1 ±1.3	0.056
Apo A1 (mg/dl)	136.2 ±3.1	134.3 ± 4.4	0.738
Apo B (mg/dl)	72.4 ± 5.1	72.3 ±3.4	0.984
hs-CRP (mg/L)	1.82 ± 0.34	2.31 ± 0.55	0.446
Glucose (mg/dl)	95.3 ±1.9	95.3 ±2.4	0.997
HbA1c (%)	5.8 ± 0.07	5.9 ± 0.08	0.315
Insulin (mU/l)	8.38 ± 0.91	9.41 ± 1.0	0.453
HIRI	1075 ± 107	993 ± 99	0.575
MISI (x 10^2)	2.18 ± 0.57	2.08 ± 0.32	0.884
DI	0.95 ± 0.09	0.79 ± 0.07	0.160
ISI	4.31 ± 0.62	3.84 ± 0.44	0.543
IGI	**1.09 ± 0.11**	**0.73 ± 0.09**	**0.012***
HOMA-IR	2.60 ± 0.27	2.45 ± 0.25	0.674

Subjects were classified according to T2DM-development status after 3 years of follow-up. Values expressed as mean ± standard error. ***BMI***, Body mass index; ***c-HDL***, High density lipoprotein; ***c- LDL***, Low density lipoprotein; ***TG***, Triglycerides; ***hs-CRP***, High sensitivity C-reactive protein; ***HbA1c***, Glycosylated hemoglobin; ***HIRI***, Hepatic insulin resistance index; ***MISI***, Muscle insulin sensitivity index; ***ISI***, Insulin sensitivity index; ***IGI***, Insulinogenic index; ***DI***, Disposition index; ***HOMA-IR***, Homeostasis model assessment- insulin resistance; * $p < 0.05$. Variables were calculated by ONE-WAY ANOVA analysis using SPSS (now PASW Statistic for Windows (version 21.0)) (IBM. Chicago, Illinois). Subjects did not develop T2DM or pre-DM (**non-T2DM**); Subjects developed T2DM after the follow-up period (**incident-T2DM**).

Metabolites identified in plasma of the whole population studied

We identified 456 metabolites distributed between 23 families, which suggests close chemical heterogeneity, as represented in **Figure 1**.

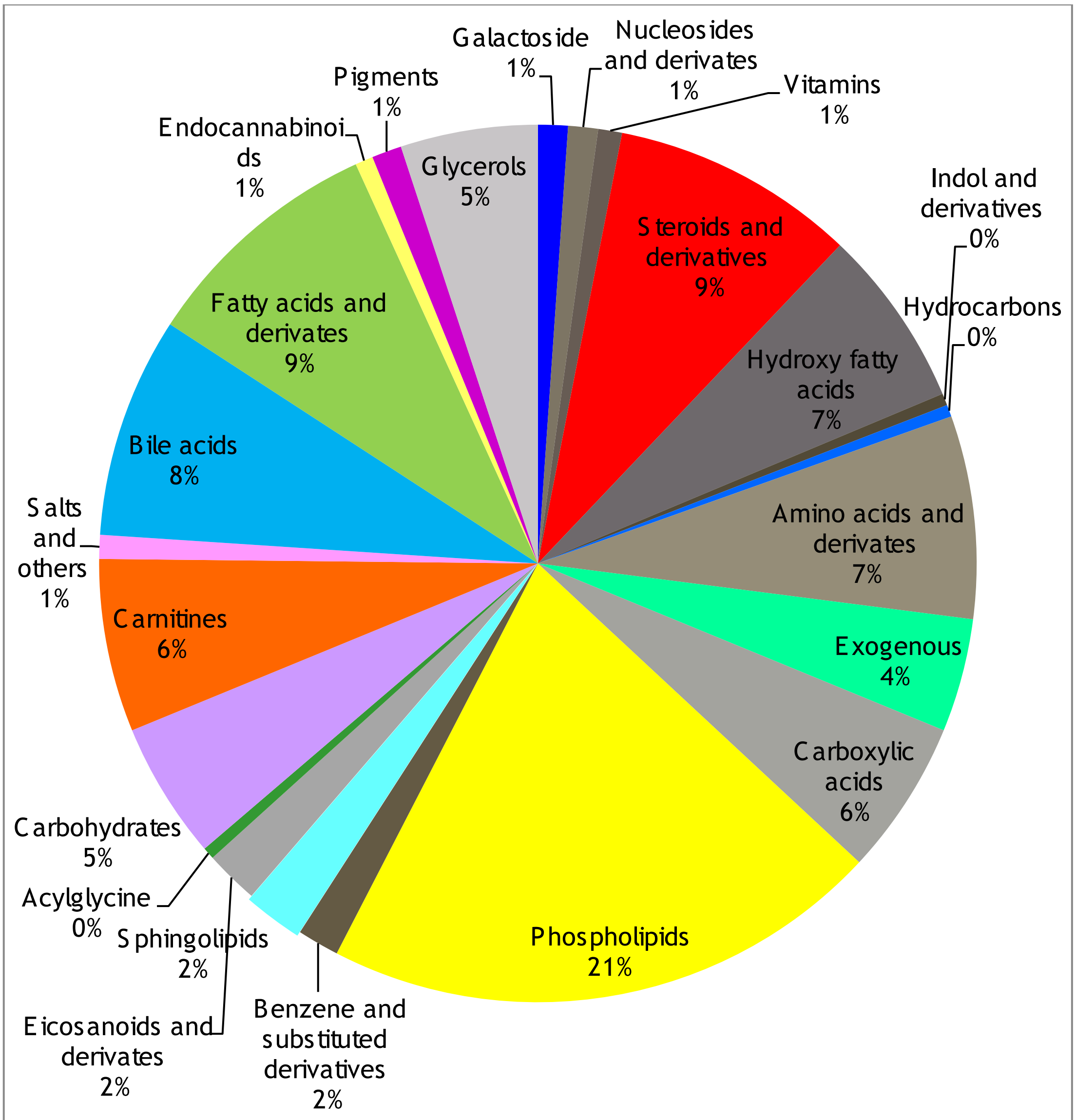

Figure 1: *Plasma metabolites identified in the population studied*

Multivariate analysis by PCA, PLS-DA and OPLS-DA

While PCA did not yield statistically significant results, the PLS-DA showed differences between Incident-T2DM and non-T2DM in the fasting (R^2X=0.166; R^2Y= 0.722; Q^2=0.162) and the postprandial state (R^2X=0.148; R^2Y= 0.759; Q^2=0.19), respectively (**Figure 2**). Moreover, based on the Variables Importance Projection analysis (VIP), 51 metabolites were identified with VIP scores >1.5 in the fasting state (**Table 2**) and 54 metabolites in the postprandial state (**Table 3**), which highlights its relevance in differentiating between Incident-T2DM and non-T2DM patients.

The metabolites that appear only in the fasting analysis are highlighted in red (**Table 2**) and belong mostly to the glycerophosphocholines family (such as LysoPC(14:0) or LysoPC(20:2)); there are also amino acids (isoleucine), phenols and derivatives (benzenediol), tetrapyrroles and derivatives (dihydrobiliverdina) and triterpenoids families (carboxymethyl-cholestaneol).

On the other hand, the metabolites exclusive to the postprandial analysis are highlighted in yellow (**Table 3**) and belong mostly to fatty acids and conjugates (such as myristic, palmitic or stearic acids) and fatty acid ester families (such as dodecanoylcarnitine or tetradecenoylcarnitine), in addition to the families of bile acids, alcohols and derivatives (glycochenodeoxycholic acid), amino acids (arginine), glycerophosphocholines (PC), glycerophosphoglycerols (PG), hydroxysteroids (cortol, cortolona and tetrahydrodeoxycorticosterona), lineolic acids and derivatives (HODE and HpODE), steroidal glycosides (such as glycochenodeoxycholic acid glucuronide or estriol-glucuronide) and sulfated steroid families (such as dihydroxyandrosterone).

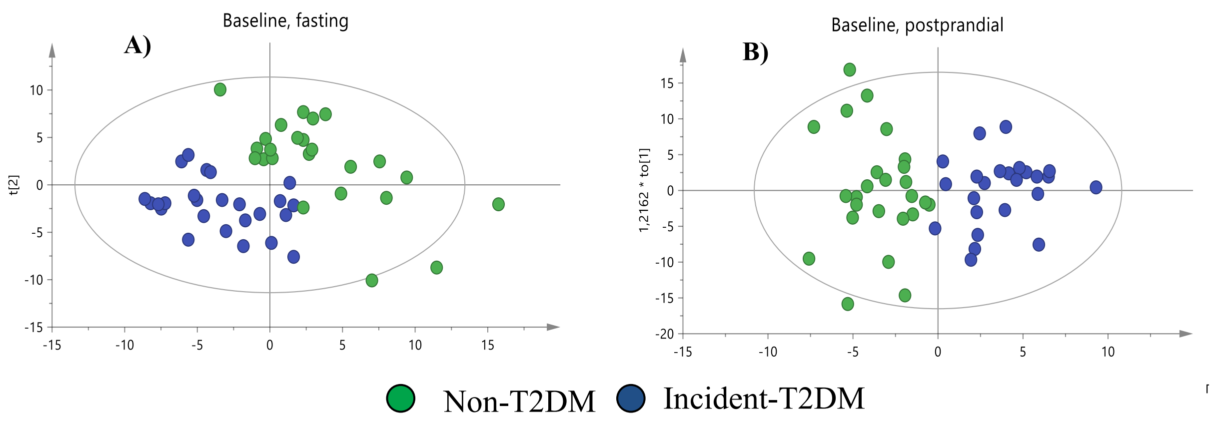

***Figure 2**: Principal Component Analysis (PCA) at baseline. A) Partial Least Square analysis discriminant analysis (PLS-DA) in the fasting state B) Orthogonal Partial Least Square discriminant analysis (OPLS-DA) after 4 hours of fat overload (postprandial state)*

Table 2: *Metabolites with VIP score >1.5 after the PLS-DA analysis in the fasting state.*

Metabolites	VIP
Dideoxy-imino-arabitol	2,73643
PC(38:4)-1	2,56743
LysoPC(22:5)-1	2,52281
LysoPC(16:1)-1	2,46719
PC(36:3)-1	2,40229
LysoPC(14:0)-1	2,34604
Sphingosine-1-phosphate	2,30885
LysoPC(20:2)	2,28889
Oleoyl triptophan	2,26144
LysoPC(14:0)-2	2,25497
LysoPC(18:3)-1	2,20762
Palmitoyl tryptophan	2,18544
PC(34:1)-2	2,18085
LysoPC(16:0)-1	2,14687
Isoleucine/leucine	2,14027
Hydroxy-methylglutamate	2,00073
LysoPC(17:1)	1,99568
Dihydrobiliverdin	1,97562
LysoPC(22:6)-2	1,96462
Hydroxyperoxy-C18:2	1,93767
Carboxymethyl-cholestaneol	1,89856
Cysteinylglycine disulfide	1,88107
O-Methylthreonine	1,85494
LysoPE(20:3)-3	1,85433
Methoxycinnamaldehyde	1,81161
LysoPC(20:0)	1,79316
Benzenediol	1,78891
Norcholestahexol isomer 1	1,78566
Cholic acid isomer 2	1,77773

Metabolites	VIP
Marcador de inflamacion C18:2	1,74151
Cholestane-tetrol isomer 1	1,72821
Anandamide-18:3	1,70426
C24:2	1,69975
MG(22:2)	1,67441
Hydroxytridecanoic acid	1,66712
PI(36:0)	1,64757
NeuAcalpha2-3Galbeta-Cer(d18:1/18:0)	1,63847
PC(O-34:3)/PC(P-34:2)	1,63525
delta-Tocopherol	1,62983
Undecanoylglycine	1,61207
PC(38:5)-1	1,61129
Palmitoleoyl-ethanolamine	1,60709
LysoPC(18:0)-1	1,59506
LysoPE(20:3)-1	1,59017
Isopentenyladenine	1,58999
Tocopherol acetate isomer 1	1,58066
C24:3	1,5508
Tyrosine	1,53477
MG(22:4)	1,51815
Homogesterone	1,50378

Table 3: *Metabolites with VIP score >1.5 after the OPLS-DA analysis in the postprandial state.*

Metabolites	VIP
Dideoxy-imino-arabitol	2,78982
Hydroxy-methylglutamate	2,64522
cis-5-Tetradecenoylcarnitine	2,43213
Hexadecanedioic acid mono-L-carnitine ester	2,33354
Glycochenodeoxycholic acid glucuronide isomer 1	2,29432
Hydroperoxy-C18:3	2,25798
HODE	2,1922
Glycochenodeoxycholate sulfate	2,14444
Myristic acid (C14:0)	2,13869
HpOTrE/HpODE	2,12807
O-Methylthreonine	2,10125
PC(34:2)	2,08719
PC(38:4)-2	2,01298
Palmitic acid (C16:0)	1,9795
Palmitoyl tryptophan	1,97186
5-a-Androstane-3a-ol-17-one sulfate/Dihydrotetosterone sulfate/Etiocholanolone sulfate	1,96712
PC(P-36:4)	1,94193
PG(22:0)	1,88848
Dihydroxyandrosterone sulfate	1,88471
Cysteinylglycine disulfide	1,88232
Dodecanoylcarnitine	1,87703
C16 sphingosine	1,84139
LysoPC(22:5)-2	1,80359
Coprocholic acid	1,76916
Norcholestahexol isomer 1	1,76199
MG(22:2)	1,75695

Metabolites	VIP
Eicosanoid 2	1,74499
Cholic acid glucuronide	1,74131
Cortolone	1,73895
Decanoylcarnitine	1,72905
Dodecanoylcarnitine	1,72624
Marcador de inflamacion C18:2	1,69097
Oleoyl triptophan	1,6807
C17:1	1,67934
Arginine	1,66916
Cholic acid isomer 2	1,66343
Norcholestahexol isomer 2	1,66291
Tetrahydrocorticosterone/Tetrahydrodeoxycortisol	1,61001
C24:3	1,60555
Stearic acid (C18:0)	1,58576
PC(36:3)-2	1,58242
Estriol-glucuronide-hormone 2	1,57784
Cholestane-tetrol isomer 1	1,56831
PG(20:1)	1,56655
Dihydroxy-3-oxo-4-cholenoic acid	1,56177
Tetradecenoylcarnitine	1,55993
Cholic acid isomer 1	1,54683
Anandamide-18:3	1,5396
Allo/Cortol	1,53812
Hexanoylcarnitine	1,51808
Octanoylcarnitine	1,51154
C24:2	1,50924
Undecanoylglycine	1,50134
Estriol-glucuronide-hormone 1	1,50089

Received Operating Characteristic (ROC) curve analysis

To evaluate the potential of the plasma metabolites as predictive biomarkers of T2DM development, we built a ROC curve with the metabolites with a VIP score > 1.5 in the PLS-DA and OPLS-DA analyses. Thus, we included the full metabolite dataset (51 metabolites) from the PLS-DA analysis in the fasting state, and we obtained an area under the curve (AUC) of 0.794, (95% CI = 0.649-0.961), with a sensibility = 0,808, specificity= 0.731 and accuracy= 72.8% (**Figure 3**). Then, we performed the ROC analysis with the metabolites with a VIP score > 1.5 from the OPLS-DA analysis in the postprandial state (54 metabolites). The ROC analysis shows an AUC=0.849, (95% CI = 0.678-0.960), sensibility = 0,814, specificity= 0.727 and accuracy= 74.1% (**Figure 4**).

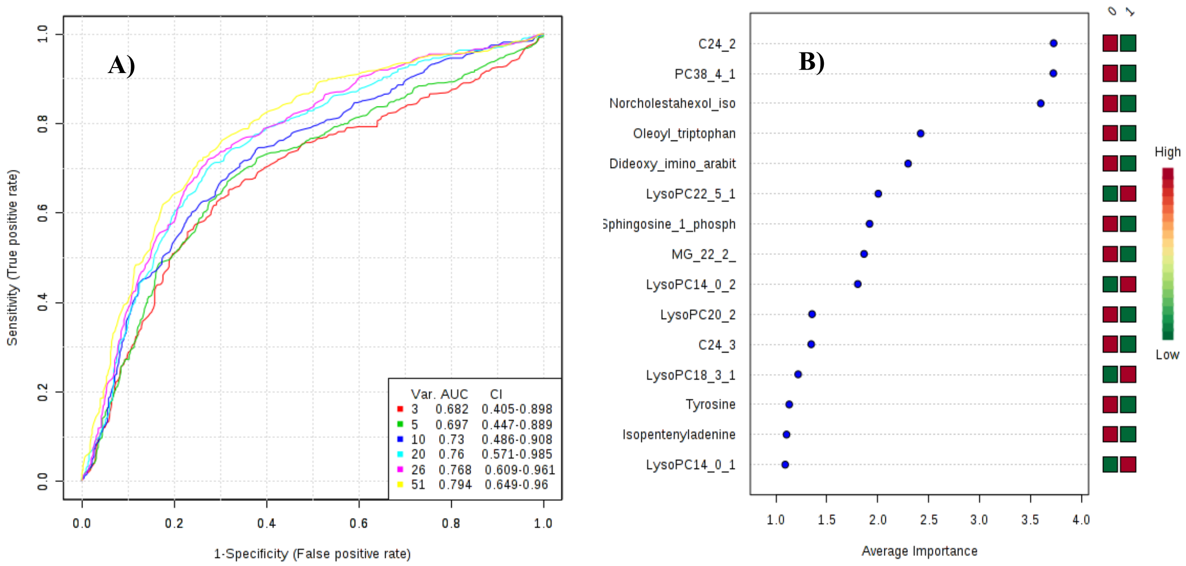

Figure 3: *ROC (Receiver Operating Characteristics) curves analysis at baseline. A) ROC curve analysis with the metabolites obtained in the PLS-DA analysis at fasting with a VIP score> 1.5. B) Average importance analysis of the metabolites included in the ROC curve. 0, Non-T2DM and 1, Incident-T2DM.*

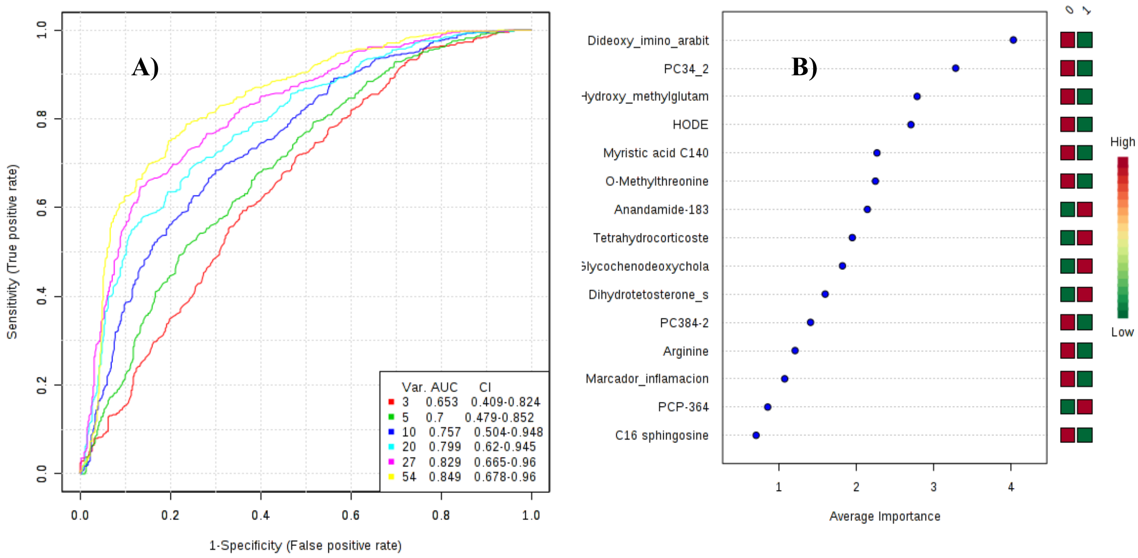

Figure 4: *ROC (Receiver Operating Characteristics) curves analysis at baseline. A) ROC curve analysis with the metabolites obtained in the OPLS-DA analysis at postprandial with a VIP score> 1.5. B) Average importance analysis of the metabolites included in the ROC curve. 0, Non-T2DM and 1, Incident-T2DM.*

According to the average importance analysis score, low levels of C(24:2), PC(38:4) and Norcholestahexol in the fasting state (**Figure 3B**); and low levels of dioxy-imino-arabitol, PC (34:2), hydroxymetylglutamate, HODE and myristic acid in the postprandial state, have the potential to differentiate between Incident-T2DM and non-T2DM subjects (**Figure 4B**).

DISCUSSION

Our study suggests that in both the fasting and postprandial state, the metabolomic profile allows us to differentiate between subjects who develop (Incident-T2DM) and do not develop (non-T2DM) type 2 diabetes mellitus after 3 years of follow-up. In fact, the ROC analysis showed that a specific plasma metabolite profile could have potential as a biomarker to predict T2DM development. Our results show an AUC of 0.794 (sensibility = 0,808, specificity= 0.731 and accuracy= 72.8%) in a model made with the metabolites identified in the fasting state with a VIP score>1.5, and an AUC of 0.849 (sensibility = 0,814, specificity= 0.727 and accuracy= 74.1%) in the postprandial analysis. In general, the results (PLS-DA, OPLS-DA and ROC analyses) showed that the plasma metabolites identified in the fasting state belong mostly to glycerophosphocholines family and those from the postprandial analysis to the fatty acids family.

Dyslipidemia plays a key role in the development of insulin resistance [327]. Although this is generally manifested by an increase in circulating free fatty acids (FFAs) and triacylglycerides (TGs), it is likely that the content and composition of other lipid species is also altered, thus contributing to the pathogenesis of T2DM. In our study, the LysoPC(14:0) and the LysoPC(20:2) are able to differentiate between Incident-T2DM and non-T2DM in the fasting state and, therefore, could have potential as early biomarkers of T2DM development. The Lysophosphatidylcholines (LPC or LysoPC) are important signalling molecules with diverse biological functions and are involved in the regulation of cellular proliferation, tumour cell invasion and inflammation in obesity [328-331]. Increased levels of LPCs are therefore associated with obesity [332] and T2DM [333]. Other studies have demonstrated that plasma LPC levels are reduced in individuals with impaired glucose tolerance in a model of steatohepatitis [334] and in a high fat-fed model of obesity, both in mice [335].

In addition, our study also showed that other kinds of glycerophosphocholines (such as PC (36:2) and PC (36:4)), myristic acid, HODE, arginine and glycochenodeoxycholic acid are able to differentiate between Incident-T2DM and non-T2DM in the postprandial state, according to the VIP and average importance scores obtained from the OPLS-DA and ROC analyses. Previous studies have shown that myristic acid increases levels of diacylglycerol kinase (DGK) δ, an enzyme that converts diacylglycerol to phosphatidic acid [336, 337] and improves glucose uptake *in vitro* [338]. It is known that glucose-induced insulin resistance is associated with a long-term increase in intracellular diacylglycerol [339]. In addition, Chibalin et al. revealed that decreased levels of DGKδ are closely linked to high glucose-induced insulin resistance, which increases the severity of type 2 diabetes [340]. Moreover, results from the study by Takato et al. suggest that chronic oral administration of myristic acid improves hyperglycemia by decreasing insulin-responsive glucose levels in a model of congenital T2DM in mice [341].

In our study, amino acids such as arginine are also part of the group of metabolites that could be used as biomarkers to differentiate between groups of subjects at risk of type 2 diabetes mellitus development. Under conditions of low arginine, nitric oxide synthase (NOS) is uncoupled, leading to reactive oxygen species synthesis and subsequent oxidative stress instead of nitric oxide [342], which is critical for a normal vasomotor and endothelial function. Moreover, reduced levels of NO are associated with insulin resistance. Activation of NO synthase (NOS) increases the blood flow to insulin-sensitive tissues (i.e. skeletal muscle, liver, adipose tissue), and its activity may be impaired by hyperglycemia and insulin resistance [343, 344].
Sangeeta et al. suggest that insulin resistance in T2DM may contribute to reduced NOS activity by generating methylated arginine (asymmetric dimethylarginine, ADMA), while hyperglycemia contributes to increased activity of arginase and reactive oxygen species levels, which further inhibit NOS activity. In addition to its role as a substrate for NOS, arginine has vasodilatory, anti-inflammatory and anti-atherosclerotic properties [345, 346]. Increased oxidative stress and concentrations of asymmetric dimethylagrinine (ADMA, or methylated arginine), added to a competitive inhibitor of nitric oxide synthase, in turn further reduce NO bioavailability and are predictive of cardiovascular risk [347]. Previous studies have shown the relationship between increased ADMA, oxidative stress and endothelial dysfunction in type 2 diabetes and obesity [347, 348]. Thus, the low plasma levels of arginine observed in our study in the Incident-T2DM suggest the potential value of arginine as predictive biomarker of T2DM development.

Additionally, in our study, differences in bile acid levels, such as glycochenodeoxycholic acid, could also be used to identify subjects at risk of developing DMT2. Bile acids are important endocrine molecules that play a key role in regulation of the body's response to food intake [349]. They interact with the nuclear farnesoid X receptor (FXR) and G-protein coupled receptor (TGR5) [350] to regulate glucose, lipid and energy homeostasis. Bile acids also control lipid solubilisation and absorption in the L-cells of the intestinal epithelium [2]. In addition, TGR5 activation by bile acids stimulates the release of gut hormones such as glucagon-like peptide-1 (GLP-1) [351], improving the glucose homeostasis [352]. In fact, previous studies have shown that plasma glycochenodeoxycholic acid and glycodeoxycholic acid concentrations correlate with GLP-1 after a mixed meal test [353]. The postprandial release of the incretin GLP-1 is impaired in type 2 diabetes mellitus [354], and HbA1c inversely correlates with maximal GLP-1 release [355]. In pancreatic beta cells, TGR5 and FXR activation enhances insulin secretion [356, 357], while in the liver, FXR activation may inhibit gluconeogenesis.

In this line, increased hepatic glucose production is an essential feature of fasting hyperglycemia in type 2 diabetes [358]. Glycogen synthase (GS) and glycogen phosphorylase (GP) are the key enzymes that control the synthesis and degradation of glycogen, respectively. The effects of 1,4-dideoxy-1,4-imino-D-arabinitol (DAB) on GP have been investigated in vitro, in primary cultured rat hepatocytes [359] and in vivo, using ob/ob mice [360, 361]. Latsis and co-workers [359] concluded that DAB is an allosteric inhibitor of GP, while Fosgerau et al [360] suggested that DAB acts as a non-competitive inhibitor of the hepatic glycogen breakdown in vivo and displayed an anti-hyperglycemic effect, which was most pronounced in obese mice. In our study, we found that the DAB was a very important metabolite in fasting and in the postprandial state in the differentiation between Incident-T2DM and non-T2DM patients, with lower plasma levels in subjects who develop the disease.

Lipid peroxidation is involved in the underlying mechanisms of several disorders and diseases such as cardiovascular diseases, cancer, neurodegenerative diseases, even aging and T2DM [362, 363]. Hydroxyoctadecadienoic acids (HODEs), formed from linoleic acid (LA), a polyunsaturated omega-6 fatty acid, by a free-radical-mediated oxidation mechanism, have recently received special attention as a potential biomarker [364]. Some studies have described the detection of these molecules, for example, 9-hydroxy LA in the erythrocyte membranes of diabetic patients [365] and hydroxy fatty acid in atherosclerotic patients [366]. Furthermore, the usefulness of HODE as a biomarker of oxidative stress has also been reported [362, 364, 367, 368]. Umano et al. [369] showed that 10- and 12-(Z,E)-HODE, although not 9- and 13-HODE, were closely correlated with clinical values for diabetes and had potential to diagnose these diseases. These authors have also demonstrated [363], and later validated [370], that multiple markers including insulin, leptin/adiponectin, and 10- and 12-Z,E-HODE/LA can be used for the early diagnosis of diabetes. In our study, HODE was a highly significant metabolite in the postprandial state in the differentiation between Incident-T2DM and non-T2DM patients and, therefore, could also be used to identify subjects at risk of developing the disease.

In conclusion, our results, taken all together, suggest that the plasma metabolite profile is altered in T2DM patients several years before the onset of the disease. Certain metabolites, including lysophosphatidylcholines, aminoacids such as arginine, myristic acid, HODE, and bile acids, such as glycochenodeoxycholic acids, could become valuable biomarkers to predict the development of T2DM.

VII. CONCLUSIONS

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The plasma levels of certain miRNAs, LPS and metabolites profile could have a valuable potential as biomarkers to evaluate the probability and predict the develop of type 2 diabetes mellitus in high-risk patients with coronary heart disease. This main conclusion is supported by the findings corresponding to the three papers of this thesis:

1. An elevated postprandial endotoxemia precedes the development of T2DM in patients with coronary heart disease and probably plays a role by promoting inflammation-induced insulin resistance and/or beta-cell dysfunction. In addition, our results also support the role of LPS plasma levels as a predictor biomarker and as a pathogenic factor in T2DM development. *Conclusion from paper 1.*

2. *miR-9*, *miR-28-3p*, *miR-29a*, *miR-30a-5p*, *miR-103*, *miR-126*, *miR-150*, *miR-223*, and *miR-375* plasma levels added to HbA1c could become a valuable new tool for assessing the early risk of T2DM in clinical practice to prevent the development of this disease. *Conclusion from paper 2.*

3. The plasma levels of *miR-150, miR-30a-5p, miR-15a*, and *miR-375* are deregulated years before the onset of T2DM and pre-DM and could be used to evaluate the risk of developing the disease, which may improve prediction and prevention among individuals at high risk of T2DM. *Conclusion from paper 3.*

4. The metabolic profile is altered in T2DM patients several years before the disease onset. Certain metabolites including lysophosphatidylcholines; aminoacids such us arginine; myristic acid; HODE; and bile acids such us the glycochenodeoxycholic could become valuable biomarkers in the prediction of the T2DM development.

VIII. REFERENCES

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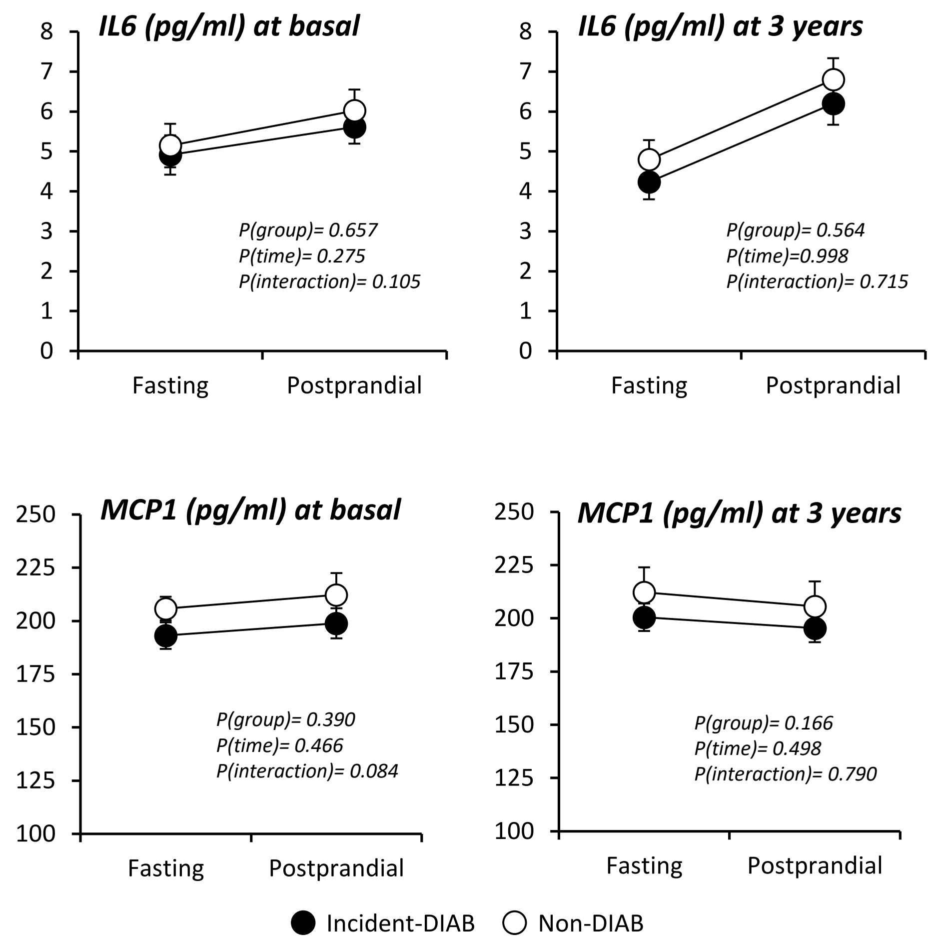
IX. SUPPLEMENTAL INFORMATION

PAPER 1



Supplemental Figure 1. Fasting and postprandial levels of the inflammatory cytokines IL6 and TNF-a. Mean (±S.E.M.) of IL6 (ng/mL) and MCP1 (ng/mL) plasma levels at 12-h fasting and after the administration of the mixed meal at baseline and at 3 years of follow-up. ANOVA for repeated measures p-values adjusted by age, gender and BMI.

PAPER 2

miRNA	amplified in plasma samples in our study	Fluid, tissue or cell type	Model	References
miR-103 and 107	yes		Ob/ob mice and diet-induced obese (DIO)	[1] Trajkovski, et al.
		Pancreatic cell line MIN6	mouse	[2] Foley,et al.
		Serum	human	[3] Xu, et al.
		Liver C57BL/6J	mouse	[1] Trajkovski, et al.
miR-126	yes	Plasma	Human and hyperglycemic Lep(ob) mice	[4] Zampetaki, et al.
		Serum	Human	[5] Liu, et al.
		SK-Hep1 hepatocytes	Human	[6] Ryu. et al.
			Mouse ob/ob	[7] Liang, et al.
miR-143	yes	C2C12 cells		[8] Gallagher, et al.
		Muscle	Human	
		Liver	mice	[9] Jordan, et al.
miR-144	yes	Plasma	Human, rat	[10] Karolina, et al
		C2C12 cells		[8] Gallagher, et al.
		Muscle	Human	
miR-145	yes	HepG2 cells		[11] Kang, et al.
		Islets	mice	
miR-150	yes	Plasma	Human, rat	[10] Karolina, et al
		b-cell	mice	[12] Xiao, et al.
		Plasma	Human	[12, 13] Devaux, et al.
		C57BL/6J	mice	[14] Li, et al.
		THP-1 cells	Human	
		Microvesicles	Human	
miR-15a	yes	Plasma	Human and hyperglycemic Lep(ob) mice	[4] Zampetaki, et al.
miR-182	yes	Plasma	Human, rat	[10] Karolina, et al
miR-192	< 80 % of samples	Plasma	human, rat.	[10] Karolina, et al.
		3T3-L1 Adipocytes	Rat	[15] He, et al.
miR-21	< 80 % of samples	Wound tissues	KKAY mice (type-2 diabetic strain)	[16] Madhyastha, et al.
		3T3-L1 Adipocytes	mice	[17] Ling, et al.
miR-223	yes	Plasma	Human and hyperglycemic Lep(ob) mice	[4] Zampetaki, et al.
		Macrophages	Mice	[18] Zhuang, et al.
		Serum	Human	[19] Pescador, et al.
		Cardiomyoctes	rat	[20] Lu, et al.
miR-28-3p	yes	Plasma	Human and hyperglycemic Lep(ob) mice	[4] Zampetaki, et al.
miR-29a	yes	Islets	Human; Db/db mice; NOD mice	[21, 15, 22-25] Bagge, et al.; Yang, et al.; Liang, et al.; Roggli, et al; He, et al.; Herrera, et al.
		Myocytes and GLUT4myc myocytes	Rat	
		Islets and plasma	db/m, db/db and DIO mice	
		Adipocytes 3T3-L1	mouse	
		Plasma	human, rat.	[10] Karolina, et al.
miR-30a5-p	yes	INS-1 cells	Rat.	[26] Kim, et al.
miR-30d	<80 % of samples	Plasma	human, rat.	[10, 4] Karolina, et al.; Zampetaki, et al.
		MIN6 cells	mouse	[27] Tang, et al.
		Pancreatic b cells	mouse	
miR-320	yes	Plasma	Human, rat	[10] Karolina, et al
miR-33a	no	HepG2 and Huh7 cells	human	[28] Dávalos, et al.
miR-375	yes	MIN6 and TC1 cells		[29] Poy, et al.
		NIT1 cells		[30, 31] Li, et al.; Xia, et al.
		INS-1E cells and primary rat islets	Goto-Kakizaki (GK) rats.	[32] El Ouaamari, et al.
		Pancreatic islets	Human, mice.	[33] Poy, et al.
		Serum	Human.	[34, 35] Kong, et al.; Higuchi, et al.
miR-657	no	Hep G2 cells	Human	[36] Lv, et al.
miR-7	yes	Pancreatic islets	Human, mouse	[37] Latreille, et al.
		Islets	mouse	[38] Wang, et al.
miR-9	yes	INS-1E and MIN6B1	Rat, mouse	[39] Plaisance, et al.
		beta-cell line MIN6B1	mice	[40] Lovis, et al.
miR-96	no	beta-cell line MIN6B1	mice	[40] Lovis, et al.
miR-let7	< 80 % of samples		mice	[41] Frost and Olson.
		3T3-L1 Adipocytes	Human, mouse	[42, 43] Sun, et al.; Wei, et al.

Supplementary table 1. Bibliographic search of miRNAs related to T2DM.

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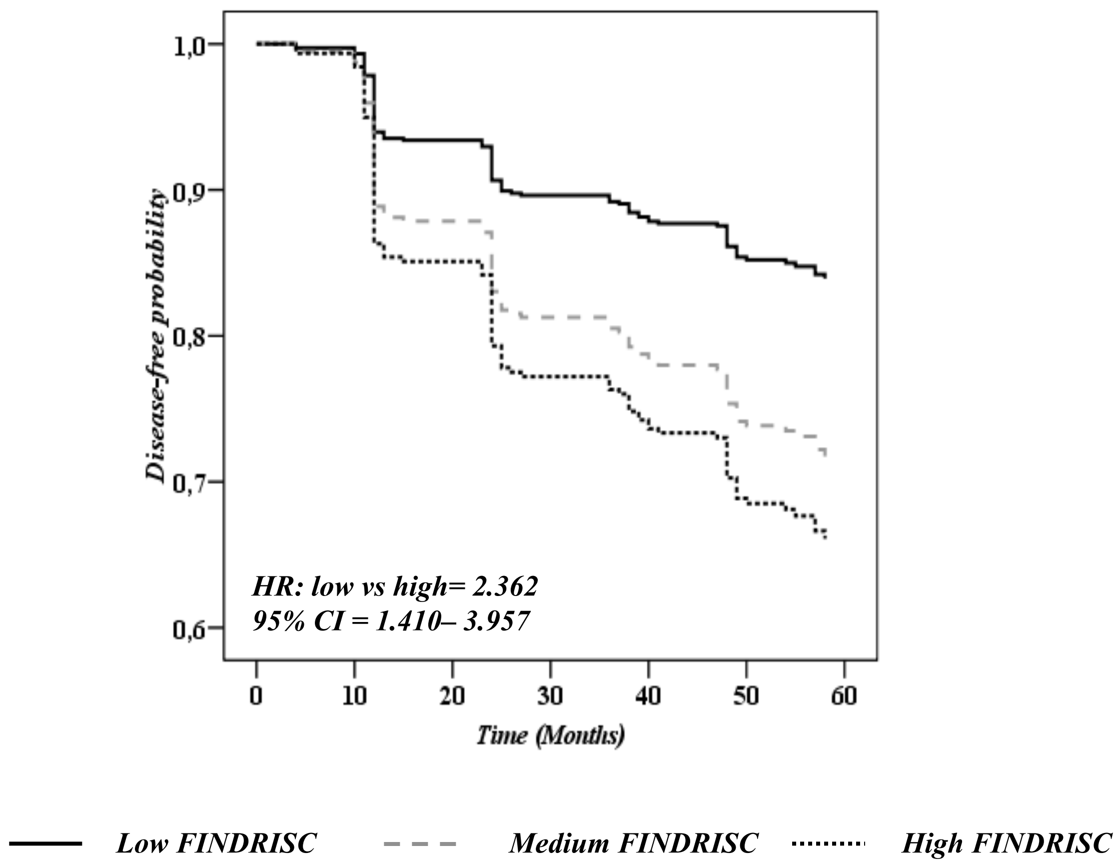
Model	*AUC*	*95% CI*	*Sensibility*	*Specificity*	*Accuracy %*
15 miRNAs	0.8487	0.808-0.889	0.796	0.774	76.9
9 miRNAs target	0.8178	0.771-0.864	0.758	0.779	77.4
9 miRNAs target and HbA1c	**0.8342**	0.790-0.878	0.776	0.809	80.0
9 miRNAs target + HbA1c + body weight (without adjusting by covariables)	**0.8212**	0.776-0.866	0.633	0.819	77.2
9 miRNAs target + HbA1c + waist circumference (without adjusting by covariables)	**0.8234**	0.778-0.868	0.610	0.814	77.9
Clinical parameters	**0.7354**	0.674-0.796	0.702	0.698	69.9
Insulin sensitivity/resistance indexes and classical parameters	0.7697	0.713-0.826	0.663	0.809	77.5
9 target and FINDRISC	0.8123	0.766-0.860	0.788	0.723	73.8
9 target, FINDRISC and HbA1c	0.8293	0.785-0.874	0.796	0.774	77.9
Insulin sensitivity/resistance indexes, classical parameters and FINDRISC	0.7647	0.709-0.820	0.663	0.755	73.3

Supplementary Table 2. Predictive models performed in our study by Operating Curve Analysis (ROC). We built models including all the miRNAs measured together with classical parameters (fasting glucose, 2-h glucose, HbA1c), beta cell function and insulin sensitivity/resistance indexes (IGI, ISI, DI, HIRI, MISI) and FINDRISC score. *AUC*, Area under curve; CI, Confidence interval; *HbA1c*, Glycosylated hemoglobin. All the models were adjusted for those covariables that were allowed avoiding over estimating information, the set of covariables included: diet, age, gender, BMI, HDL, TG, Hba1c and waist circumference.



Supplementary Figure 1. Disease-free analysis through a Cox regression model based on FINDRISC. Data represent subjects of our study categorized by tertiles of FINDRISC score, low risk (T1), medium risk (T2) and high risk (T3). The analysis was carried out through SPSS (now PASW Statistic for Windows (version 21.0)) (IBM. Chicago, Illinois).

CORDIOPREV study 1002 patients

540 type 2 diabetes patients	462 non type 2 diabetes patients
43 patients developed type 2 diabetes	**First year follow-up**
24 patients developed type 2 diabetes	**Second year follow-up**
11 patients developed type 2 diabetes	**Third year follow-up**
19 patients developed type 2 diabetes	**Fourth year follow-up**
10 patients developed type 2 diabetes	**Fifthyear follow-up**
107 Incident type 2 diabetes patients	355 non type 2 diabetes patients

Supplementary Figure 2. Flow chart of the study design.

X. ANNEXES

FIBICO
Fundación para la Investigación Biomédica de Córdoba

D. ÁLVARO GRANADOS DEL RÍO, GERENTE DE LA FUNDACIÓN PARA LA INVESTIGACIÓN BIOMÉDICA DE CÓRDOBA (FIBICO), CON DOMICILIO EN LA AVDA. MENÉNDEZ PIDAL, S/N DE CÓRDOBA,

CERTIFICA

Que Doña. **ROSA JIMÉNEZ LUCENA**, con DNI 31001276-J es coinventora de la Patente "Modelo predictivo para predecir el desarrollo de diabetes mellitus tipo 2 usando miARNs" con número de solicitud P201830540, solicitada ante la Oficina Española de Patentes y Marcas el cuatro de junio de 2018 por sus titulares, el Servicio Andaluz de Salud, la Universidad de Córdoba y el Consorcio Centro de Investigación Biomédica en Red, M.P.

Y para que así conste y tenga los efectos oportunos, firmo el presente certificado en Córdoba, a doce de septiembre de dos mil dieciocho.

Fdo.: D. Álvaro Granados del Río
Gerente del FIBICO

C.I.F.: G-14825277

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Estudio de los perfiles de expresión de miRNAs como posibles biomarcadores en el diagnóstico temprano de diabetes mellitus tipo 2

Presentada por los autores

Rosa Jiménez Lucena; Oriol Alberto Rangel Zúñiga; Javier López Moreno; Anabel Jiménez; María José Zarzuelo; Ruth Blanco Rojo; Carmen Marín Hinojosa; José López Miranda Hospital Virgen de la Victoria. Málaga. España. Ibermutuamur. Madrid. España

INSTITUTO MAIMÓNIDES DE INVESTIGACIÓN BIOMÉDICA DE CÓRDOBA, (1) UNIDAD DE LÍPIDOS Y ATEROSCLEROSIS/INSTITUTO MAIMÓNIDES DE INVESTIGACIÓN BIOMÉDICA DE CÓRDOBA (IMIBIC)/HOSPITAL UNIVERSITARIO REINA SOFÍA/UNIVERSIDAD DE CÓRDOBA, CÓRDOBA, ESPAÑA/CIBER FISIOPATOLOGÍA OBESIDAD Y NUTRICIÓN (CIBEROBN)/INSTITUTO DE SALUD CARLOS

Juan F. Ascaso Gimilio
Presidente S.E.A.

Granada, 20 de mayo de 2016

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"Identificación de metabolitos plasmáticos como potenciales biomarcadores en el diagnóstico temprano de diabetes mellitus tipo 2"

Presentada por los autores

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Xavier Pintó Sala
Presidente SEA

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Sociedad Española de Arteriosclerosis

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GIRONA 2018

"MiRNAs circulantes como herramienta predictiva para evaluar el riesgo de desarrollo de pre-diabetes y diabetes mellitus tipo 2 en pacientes con enfermedad cardiovascular establecida"

Presentada por los autores

Rosa Jiménez Lucena; Irene Roncero Ramos; Juan Francisco Alcalá Díaz; Nieves Delgado Casado; Purificación Gómez Luna; Oriol Alberto Rangel Zúñiga; José López Miranda

Unidad de Lípidos y Arteriosclerosis. IMIBIC/Hospital Universitario Reina Sofía/Universidad de Córdoba, Córdoba. CIBER Fisiopatología Obesidad y Nutrición (CIBEROBN), Instituto de Salud Carlos III, Madrid, España.

Pedro Valdivielso Felices
Presidente SEA